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PRINCIPAL INVESTIGATOR: Dr. Michael Hengartner

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

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Hengartner, Michael O., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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13. ABSTRACT *(Maximum 200 words)*

The main goal of our grant application is to identify novel genes that function in apoptosis (programmed cell death), using the *C. elegans* cell death suppressor CED-9 as our starting point for an extensive yeast two-hybrid screen. In the first year of funding, we have concentrated our attention on our discovery of a specific interaction between CED-9 and CED-4, one of the proapoptotic proteins in *C. elegans*. We showed that CED-4 is likely to be a major target for CED-9 action. We have also initiated work on Apaf-1, the mammalian homolog of CED-4. We have developed a simple model for how the apoptotic machinery is regulated in *C. elegans*. This model is now being tested, both in worms and in mammals. In the coming years, we plan to concentrate our attention back on the several other CED-9-interacting clones that we isolated in our screen.

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FOREWORD

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5. INTRODUCTION

5.1 Apoptosis in breast cancer development and treatment

Apoptosis (programmed cell death) is intimately involved in the regulation of cell numbers during normal breast physiology. For example, involution of the lactating breast after weaning is mediated through extensive apoptosis of breast epithelial and endothelial cells (Strange et al., 1995; Walker et al., 1989). Smaller waves of deaths also occur during each menstrual cycle, presumably in response to variations in hormonal levels (Anderson et al., 1988; Longacre and Bartow, 1986). More recently, apoptosis has also been implicated in the development and treatment of breast tumors: it has been suggested that the acquisition of mutations that reduce the cell's ability to undergo apoptosis is a crucial event in multi-step carcinogenesis (Schulte-Hermann et al., 1995); such mutations might also be responsible for the development of resistance to chemotherapy (Lowe et al., 1994; Thompson, 1995). Consistent with these ideas, a large fraction of breast tumors show increased expression of cell death protectors and/or reduced expression of cell death promoters. Thus alterations in cell death gene activity, be it through mutation or changes in expression levels, clearly contribute to breast cancer development and resistance to therapy.

5.2 Genetic analysis of apoptosis in the nematode *C. elegans*

Programmed cell death has also been extensively studied in the nematode *C. elegans*. In this species, developmental deaths are highly reproducible: the identity of the dying cells and the time in development at which these cells die are essentially invariant among individuals. This particularity is of great use, as it allows apoptosis to be studied at the single cell level with great sensitivity (reviewed in Refs. Driscoll, 1992; Hengartner and Horvitz, 1994). Genetic dissection of programmed cell death in *C. elegans* has led to the identification of 14 genes that function in programmed cell death and that can be placed into a genetic pathway. The activities of two of these three genes, *ced-3* and *ced-4* (cell death abnormal), are necessary for programmed cell deaths to occur: Mutations that inactivate either *ced-3* or *ced-4* result in the survival of all 131 cells that normally die during hermaphrodite development (Ellis and Horvitz, 1986). A third gene, *ced-9*, is required to prevent activation of the cell death program in cells that should live: either a gain-of-function mutation in the *ced-9* gene or overexpression of wild-type *ced-9* results in the survival of cells that normally die; by contrast, mutations that reduce *ced-9* function cause many cells that normally survive to undergo programmed cell death (Hengartner et al., 1992).

5.3 Molecular conservation of the apoptotic program through evolution

Our previous work on the molecular characterization of *ced-9* has revealed that this nematode gene encodes an invertebrate member of the Bcl-2 family of mammalian cell death regulators (Hengartner and Horvitz, 1994). Even more striking than the sequence

conservation is the functional conservation between *ced-9* and *bcl-2*: human *bcl-2* can prevent programmed cell death in *C. elegans* (Hengartner and Horvitz, 1994; Vaux et al., 1992) and can also substitute for *ced-9* in animals deficient in *ced-9* function (Hengartner and Horvitz, 1994). The observation that these structurally similar genes are functionally interchangeable strongly suggests that nematodes and mammals share a common molecular pathway for programmed cell death. This genetic program for cell death presumably predates the evolutionary separation of nematodes and vertebrates and thus seems likely to be of ancient origin. Further evidence of this conservation has arisen from the study of the death protein CED-3, which was found to be an invertebrate member of the ICE family of apoptotic proteases (Kumar, 1995; Miura et al., 1993; Yuan et al., 1993). This conservation of sequence and function is of great importance, as it implies that knowledge gained about nematode programmed cell death can also be used to further our understanding of this phenomenon in humans.

5.4 Hypothesis/Research proposal

Increased resistance to apoptotic stimuli often accompanies cancer development, and can be the cause of resistance to chemotherapy (Lowe et al., 1994; Thompson, 1995). This increased resistance correlates with changes in the expression or activity - either through altered regulation or through mutation - of genes involved in the apoptotic pathway (Hoskins and Weber, 1995). However, our understanding of apoptosis, and of the genes involved in its regulation and execution is still sketchy.

In our application, we postulated that the identification and characterization of additional genes that function in breast cell apoptosis would allow us to identify additional diagnostic and prognostic markers, as well as provide new targets for therapy. We proposed to use the nematode *C. elegans* as a model system to isolate such novel cell death genes. Specifically, we proposed to identify proteins that interact with the CED-9 cell survival protein in the yeast two-hybrid system. We argued that because of its simplicity, the reproducibility of its development, and the powerful molecular genetic tools available in this species, *C. elegans* is an excellent choice for such an enterprise.

6. BODY

6.1 Research aims/Technical objectives

The central goal of our grant is to identify and characterize proteins that interact with the *C. elegans* cell death regulator CED-9. Our original research proposal contained five (5) technical objectives:

1. Identification of proteins that interact with *C. elegans* CED-9 in the yeast two-hybrid system.
2. Confirmation of the specificity and relevance of the interaction detected between CED-9 and the isolated clones by testing for interaction using an *in vitro* assay
3. Analysis and characterization of the isolated interactors, and determination of their possible role in nematode apoptosis.
4. Identification and cloning of cDNAs encoding mammalian homologs of the nematode proteins found to function in cell death.
5. Investigation of the possible involvement of the homologs in mammalian apoptosis.

We are happy to report that we have made significant progress towards all the objectives mentioned above, in particular with respect to one of our CED-9 interactor – CED-4, and its mammalian homologue Apaf-1. Our work in this area is described in section 6.2. Our work on CED-4, as well as work from many other groups, have converged over the last year in this area, and have significantly advanced our understanding of how the Bcl-2 family might regulate apoptosis. We have also made modest progress with the other clones that we isolated in our screen. The work is this area is described in section 6.3.

Finally, in section 6.4, we will evaluate our progress in view of the statement of work that we submitted with our original application.

6.2 CED-4 / Apaf-1

6.2.1 *Direct interaction between* C. elegans *cell death regulators CED-9 and CED-4*

As part of our efforts to identify proteins that interact with CED-9, we discovered that CED-9 binds very tightly to CED-4, a protein that had previously been shown to be required for apoptotic cell death in *C. elegans*. While CED-4 can not be considered to be a "novel cell death protein," we felt that this interaction was of great interest, and decided to aggressively pursue this observation. We found that CED-9's ability to bind to CED-4 correlated with its ability to prevent death, suggesting that the major function of CED-9 might be to sequester CED-4 in an inactive conformation. These observations were published in *Nature* (Spector et al., 1997). A reprint of the paper has been attached as **Appendix II**. This reprint contains all the relevant technical information and experimental details. We therefore only briefly summarize our findings below.

6.2.1.1 CED-9 interacts with CED-4 in the yeast two hybrid system

Genetic studies in *C. elegans* have indicated that CED-9 prevents cell death by antagonizing the death-promoting activities of CED-3 and CED-4 (Hengartner et al., 1992; Hengartner and Horvitz, 1994; Hengartner and Horvitz, 1994; Shaham and Horvitz, 1996). In our search for proteins that interact with CED-9, we found that full-length CED-9 binds strongly to CED-4S, the death promoting form of CED-4 (Figure 1, Table 1). This interaction, which could be detected using either CED-9 or CED-4S as the bait, is specific, as neither LexA-CED-9 nor LexA-CED-4S interacted with other proteins used as negative controls (GAL4AD-STE11 and GAL4AD-Lamin; Figure 1, Table 1).

6.2.1.2 CED-9 interacts with CED-4 in an in vitro system

To test whether the CED-9/CED-4 interaction that we observed in the yeast two-hybrid system was direct, we asked whether CED-9 and CED-4 could interact in vitro. We found that in vitro translated CED-4S protein readily bound to glutathione-S-transferase-CED-9 (GST-CED-9) fusion protein (Figure 2A, 2B). There is little binding of CED-4S and GST-CED-9 to GST-p16 and CDK4 (two non-relevant proteins used as negative controls), respectively, confirming that the CED-9/CED-4S interaction is specific (Figure 2A, 2B).

6.2.1.3 CED-9 prevents apoptotic cell death by binding to CED-4

Given the strong genetic link between *ced-9* and *ced-4*, it is likely that the direct physical interaction between CED-9 and CED-4 is biologically significant. To test this hypothesis, we asked whether there is a correlation between the ability of CED-9 to interact with CED-4 and its ability to prevent cell death. To this end, we introduced point mutations into the *ced-9* open reading frame (ORF) to recreate three CED-9 mutant proteins whose effect on cell death we had previously characterized at the genetic level: CED-9(G169E), the product of the dominant gain-of-function allele *ced-9(n1950)*, CED-9(Y149N), which corresponds to the loss-of-function mutation *ced-9(n1653*ts*)*, and Q160stop, which results in the truncated protein CED-9(1-159) and corresponds to the loss-of-function mutation *ced-9(n2077)* (Hengartner et al., 1992; Hengartner and Horvitz, 1994; Hengartner and Horvitz, 1994).

The *n1950* gain-of-function mutation results in a glycine-to-glutamate (G169E) substitution in the conserved Bcl-2 homology (BH) domain BH1, which is present in and mediates interactions between most Bcl-2 family members (Farrow and Brown, 1996). Consistent with the observation that *n1950* does not eliminate ced-9 function, we found that CED-9(G169E) interacted efficiently with CED-4S (Table 1, Figure 2C). Because the CED-9(G169E)/CED-4S interaction was not significantly stronger than the one we observed between CED-9(+) and CED-4S, n1950 is unlikely to cause a gain of function by simply increasing CED-9/CED-4 interaction strength. Rather, we suspect - based on the observation that human Bcl-2 can interact with many distinct proteins - that *n1950* affects the ability of CED-9 to interact with another, as yet unidentified partner - possibly the nematode equivalent of Bax. Alternatively, *n1950* might alter the *in vivo* regulation of the CED-9/CED-4 interaction in ways that cannot be detected in our assay systems.

In contrast, the Y149N and Q160stop loss-of-function mutations both abrogated interaction between CED-9 and CED-4 in the yeast two-hybrid system (Table 1). Q160stop also eliminated the CED-9/CED-4 interaction *in vitro*, while Y149N only reduced it (Figure 2C). The weak effect of Y149N on CED-9/CED-4 interaction in vitro is consistent with the temperature-sensitive nature of this mutation Hengartner et al., 1992, and the different

temperatures at which our assays were performed (30 °C for yeast; 4 °C in vitro). In vivo, Y149N might prevent CED-9/CED-4 interaction only at high, but not at low temperatures (we have excluded trivial explanations, such as reduced protein stability for the *n1653* temperature sensitivity, as the Y149N protein is as stable as the wild-type protein under both permissive and restrictive temperatures). The correlation between loss of ced-9 function and loss of CED-4-binding is consistent with the hypothesis that direct interaction with CED-4 is important for the ability of CED-9 to prevent cell death.

6.2.1.4 Interaction of CED-9 with CED-4 requires most of the CED-9 protein, but not the hydrophobic C-terminal domain

To identify the domain(s) of CED-9 required for interaction with CED-4, we generated a number of CED-9 deletion constructs and tested their ability to interact with full-length CED-4S in vitro (Figure 2C). Several Bcl-2 family members contain a C-terminal hydrophobic tail, which drives localization of these proteins to the outer surface of mitochondria, rough endoplasmic reticulum, and nuclear membranes. Truncations that remove this tail only slightly reduce the ability of Bcl-2 to prevent apoptosis (Hockenbery et al., 1993; Hunter et al., 1996). Similarly, we found that CED-9(1-247), which lacks the hydrophobic tail, still prevents programmed cell death when overexpressed in C. elegans (data not shown). Thus, the C-terminal tail is not essential for Bcl-2 or CED-9 function. Consistent with these observations, CED-9(1-231), which lacks the C-terminal 49 amino acids, still interacted with CED-4S (Table 1, Figure 2A, 2C). However, elimination of a further 72 amino acids that includes the BH1 and BH2 domains [CED-9(1-159) vs. CED-9(1-231)] completely eliminated the ability of CED-9 to interact with CED-4S, both in vitro and in yeast (Table 1, Figure 2C), suggesting that the BH1 and BH2 domains, previously shown to be functionally important for several Bcl-2 family members, might be involved in CED-9/CED-4 interaction. Further support for this hypothesis comes from our observation that residues 97-231 (containing the BH1, BH2, and BH3 domains) were sufficient for interaction with CED-4S (Figure 2C).

6.2.1.5 CED-9 interacts with both splice forms of CED-4

Recently, Shaham and Horvitz Shaham and Horvitz, 1996 described an alternative splice variant of ced-4, ced-4L, that is predicted to produce a protein containing 24 additional amino acids. In contrast to the major isoform (*ced-4*S), overexpression of *ced-4*L prevents death. However, both forms of *ced-4* are antagonized by ced-9 (Shaham and Horvitz, 1996), possibly explaining why *ced-9* also possesses a minor death-promoting activity Hengartner and Horvitz, 1994. Because *ced-9* antagonizes both *ced-4*L and *ced-4*S, we suspected that CED-9 would also interact with CED-4L. Indeed, we found no qualitative differences between CED-4S and CED-4L in their ability to bind to CED-9, although the CED-9/CED-4L interaction was invariably weaker (Figure 2B, 2C). Thus, the difference between *ced-4*S and *ced-4*L must lie in a differential ability to interact with or activate downstream targets, rather than in differential binding to CED-9.

6.2.1.6 Conclusions

In summary, we have demonstrated a direct physical interaction between the *C. elegans* death suppressor CED-9 and one of its known downstream targets, CED-4. This interaction is disrupted in two *ced-9(lf)* mutants, and might be mediated through the conserved BH1, BH2, and BH3 domains. While our data suggest that binding to CED-4 is important for CED-9 function, we do not know whether it is sufficient. A simple molecular

model consistent with our observations and previously described genetic data would be that CED-9-bound CED-4 is unable to promote apoptosis, and that death-promoting stimuli induce dissociation of the two proteins (see section 6.2.2 below). The conservation of the death machinery between nematodes and mammals led us to suggest that, as for *ced-3* and *ced-9*, mammals would contain one or several proteins that play a role similar to that played by *ced-4* in *C. elegans* (Spector et al., 1997). This prediction proved right, with the description by Wang and colleagues of Apaf-1, a mammalian protein involved in the activation of caspase-9 (see section 6.2.3 below). Thus, we believe that further study of the CED-9/CED-4 interact will help to elucidate how apoptosis is controlled in mammals.

6.2.2 *The apoptosome Model of programmed cell death in* C. elegans

Our work on CED-9, and the work of several other groups, have led to the emergence of a simple molecular model for the control of apoptosis in *C. elegans*, which we have developed and refined over time (Hengartner, 1997; Hengartner, 1998; Hengartner, 1998; Hengartner, 1998). This model is based on a number of protein-protein interactions that have been detected between the various *C. elegans* cell death proteins (Figure 3). Generation of a clear model of how apoptosis is controlled in *C. elegans* is of more than academic interest, as it might also shed light on the mechanism that underlies apoptosis in mammals, or at the very least will suggest experiments that will address this question.

6.2.2.1 CED-9 and CED-4 are part of a multiprotein complex – the apoptosome

In addition to the interaction between CED-9 and CED-4, which we and several other groups reported (Chinnaiyan et al., 1997; James et al., 1997; Spector et al., 1997; Wu et al., 1997), CED-4 can also interact with proCED-3, the inactive zymogen form of CED-3, and promote its proteolytic activation (Seshagiri et a.,1997; Chinnaiyan et al., 1997; Wu et al., 1997). Thus, CED-4 might act as a chaperonin or co-factor in the activation of CED-3. As for the CED-9/CED-4 interaction, the binding of CED-4 to CED-3 is crucial for its ability to promote CED-3 activation: point mutations that inactivate the proapoptotic activity of CED-4 also abolish both interaction with proCED-3 and stimulation of CED-3 activation.

As might be expected from the available genetic data, binding of CED-9 to CED-4 abolishes its ability to promote CED-3 activation (Seshagiri et a.,1997; Chinnaiyan et al., 1997; Wu et al., 1997). However, CED-9-bound CED-4 can still interact with CED-3. Thus, in normal *C. elegans* cells, all three key cell death proteins are likely to be associated together in a multiprotein complex, which has been termed the "apoptosome", that controls cell death (Figure 3). In cells that are fated to die, we propose that the complex is modified in some way, such that CED-4 becomes active. Our favorite model is that the complex dissociates as shown in Figure 2, but there is presently no experimental support for this hypothesis. Dissociation of the complex might be mediated by EGL-1, a BH3 domain-containing protein that acts genetically as an inhibitor of CED-9 and has been shown to interact directly with CED-9 (Conradt and Horvitz, 1998).

Once freed from the shackles of CED-9, how does CED-4 promote proCED-3 processing? Recently, Baltimore and colleagues reported that CED-4, in addition to its ability to interact with CED-9 and CED-3, is also able to bind to other CED-4 molecules, resulting in CED-4 oligomerization (Yang et al., 1998). Because CED-4 is able to bind at the

same time to proCED-3 and to another CED-4 molecule, this oligomerization brings together many proCED-3 molecules, resulting in a high local concentration of proCED-3. Under these conditions, the low protease activity inherent to procaspases (Muzio et al., 1998) appears to be sufficient to allow the molecules of proCED-3 to cleave and activate one another. A similar mechanism of activation, know as "induced proximity," has recently been suggested to mediate activation of caspase-8 in mammals (Muzio et al., 1998). Induced proximity might thus be a general strategy used by cells to activate caspases (Hengartner, 1998).

As might be expected, CED-9-bound CED-4 is unable to oligomerize, either because of steric hindrance or a CED-9-induced conformational change. These observations lend further support to the idea that CED-9 prevents cell death simply by binding to CED-4 and thereby keeping proCED-3 in a safe, monomeric state (Figure 3).

6.2.2.2 Testing the apoptosome model

To directly test our model, we are raising monoclonal antibodies against bacterially expressed CED-9, CED-4, and CED-3. The antibodies will be generated by the Cold Spring Harbor Laboratory Monoclonal Antibody Facility (CSHL MAb Facility), which is part of the CSH Cancer Center, of which we are member. The CSHL MAb facility has an outstanding track record of generating useful MAbs that can be used for western blotting, immunoprecipitation, and/or immunocytochemistry. Indeed, the facility has already provided us with three distinct MAbs to CED-9, all three of which appear to specifically recognize CED-9 on a western blot (Figure 4). We expect that by next year's annual report, we will have generated a significant amount of data in this area, and will have either proved or disproved our model.

We are also constructing a number of constructs that will drive the expression of green fluorescent protein (GFP) fused to CED-9, CED-4, or CED-3. These fusions should allow us to detect any subcellular redistribution of these proteins that might occur once a cell decides to die.

6.2.2.3 Is there a mammalian apoptosome?

Since all three components of the *C. elegans* apoptosome have mammalian homologs, it is worth asking whether a similar death complex might exist in mammalian cells. Recent observations suggest that this is indeed the case.

The most obvious similarity is at the level of the CED-4/CED-3 interaction (see also section 6.2.3.1 below). In a very elegant series of papers, Wang and his colleagues have purified Apaf-1, a mammalian homolog of CED-4, based on its activity to promote the activation of caspase-9 (Apaf-3), a mammalian homolog of CED-3 (Zou et al., 1997). Indeed, Apaf-1 and caspase-9 can interact directly, as do CED-4 and CED-3. However, unlike the situation in *C. elegans*, this interaction requires the presence of another protein, cytochrome c (Apaf-2). While this twist does not invalidate the concept that interaction between CED-4 family members and caspases promotes the autocatalytic activation of the latter, it clearly indicates that we do not yet have the full picture in view, and many more embellishments to the basic model are to be expected. It also brings home again the important point that once a mammalian homolog of a worm protein is identified, it is

crucial to move the study to the mammalian system, if one wants to get an exact picture of what is going on.

In addition to the interaction with caspases, a number of groups have reported that Apaf-1 can also interact with Bcl-xL, a Bcl-2 family member, suggesting that the second interaction detected in *C. elegans* is also conserved in mammals (Hu et al., 1998; Pan et al., 1998). Finally, Apaf-1, like CED-4, is capable of oligomerization (Srinivasula et al., 1998). Thus, activation of caspase-9, like CED-3 and caspase-8, might also be mediated by induced proximity (Hengartner, 1998).

6.2.3 *Mammalian homologs of CED-4*

Our findings strongly suggest that CED-4 is a major biological target of CED-9 in *C. elegans*. In our grant application, we suggested that mammalian homologs of the CED-9 interactors identified in *C. elegans* will also function in apoptosis, and proposed to identify and study such homologs. While our prediction has proved to be correct, we were somewhat beaten to the finish line regarding the existence CED-4 homologs and their involvement in apoptosis. However, much still remains to be done, and we have initiated a few lines of investigations that should prove useful to the field.

6.2.3.1 Apaf-1

Shortly after the publication of our discovery regarding the interaction between CED-9 and CED-4, which led us to search for mammalian homologs of CED-4, Wang and colleagues described their identification of a mammalian homolog, which they called Apaf-1, as an activator of caspase-9 (Zou et al., 1997; reviewed by Hengartner, 1997).

We have obtained a full-length Apaf-1 clone from Dr. Wang. We are interested in using this clone to test the hypothesis that an apoptosome also exists in mammalian cells. To this end, we have generated, in collaboration with the group of Dr. Scott Lowe, here at Cold Spring Harbor Laboratory, a number of constructs that will drive the expression of tagged Apaf-1 protein in mammalian cells. In particular, we have generated green fluorescent protein (GFP) fusions that should allow us to follow the subcellular localization of Apaf-1 in living and dying cells. We plan to transfect these constructs into COS and MCF7 cells and establish stable clones expressing various levels of Apaf-1-GFP. In case that overexpression of proapoptotic Apaf-1 turns out to be toxic to cells, we have constructed a number of retroviral constructs, which can be used to infect cells at high efficiency.

6.2.3.1 Other mammalian CED-4 homologs

So far, only one mammalian homolog of CED-4 has been found. In contrast, there are currently at least seven mammalian homologs of CED-9, and over 13 homologs of CED-3. We thus strongly suspect that additional CED-4/Apaf-1 homologs exist in mammals. Using various search tools, we are continuing to mine the publicly available databases for new homologs that might get identified via expressed sequence tags or the various genome projects. While we readily will admit that these are true "fishing expeditions," we are convinced that any additional such homologs is likely to also be involved in apoptosis, and that this line of research is warranted and a wise time investment over the long term.

6.3 Other CED-9-interacting proteins

CED-4 is but one of over a dozen proteins that we identified as interacting with CED-9 in the yeast two-hybrid system. Our work with CED-4 has absorbed much of our energy in the last year, but we have recently found time to go back to the other clones in our collection.

6.3.2 *Isolation of CED-9-interacting clones*

Although we proposed in our application to screen well over 20 million primary transformants for clones that interact with CED-9, practical considerations prompted us to stop short of this goal. First, the libraries that we proposed to screen were not as complex as we had hoped for, and one of the three libraries was not worth screening at all. Second, we had already isolated many candidates in a preliminary screen. Thus, rather than continue to screen, we decided to start analyzing the candidates in hand. From a total of 4,500,000 primary transformants screened in the yeast two-hybrid, we identified a total of 15 clones that passed all the subsequent yeast tests (secondary screen, additional positive and negative screens). These 15 clones therefore represented good candidates for encoding *bona fide* CED-9-interacting proteins.

6.3.2 *Sequence analysis of CED-9-interacting clones*

Our first order of priority was to establish the nature of these clones. To this effect, we first sequenced both ends of the insert for each clone. Because the *C. elegans* genome project nears completion, these short sequence tags are usually sufficient to uniquely identify the gene corresponding to the cDNA insert, as well as the extent of the gene present in the cDNA. Indeed, each one of our interacting clones corresponded to a previously-identified gene (Table 2). In all, our 15 interacting clones identified 11 separate genes, which we will refer to as candidate genes from this point on.

Sequence analysis indicated that several of our candidate genes have homologs in other species (Table 2). However, none of them was homologous to previously-identified cell death genes. We consider this to be good news, as our goal is to identify novel cell death genes, not just nematode homologs of previously identified mammalian cell death genes! Three of the 11 genes (A. C, and O) stood out as the proteins that they encoded contained potential BH3 domains, which have been shown to mediate interaction with Bcl-2 family members in mammals (reviewed in Adams and Cory, 1998).

6.3.3 In vivo *loss-of-function analysis via RNA-based inhibition*

To determine whether the candidate genes identified by our clones function in programmed cell death, we used the recently described technique of RNA-mediated interference (RNAi) to generate a loss-of-function phenocopy for each of the 11 genes of interest (Fire et al., 1998). While the mechanism by which RNAi works is not clear, it has been reproduced in many laboratories, including ours. We have followed the protocol

described by Fire and colleagues for these experiments, and used *apx-1* (gift of Craig Mello) as a positive control for our RNAi experiments.

We expect that if the gene is required for cell death, then its elimination should result in the presence of extra cells, which can readily be identified in the anterior pharynx (Hengartner et al., 1992). If the gene is required to protect cells from apoptosis, then its elimination would result in extra cell death, embryonic lethality, and possibly sterility of the mother (Hengartner et al., 1992)

To confirm that cell death genes can be indeed be inactivated by RNAi, we first tested our hand with *ced-3* and *ced-4*, two genes that are required for death, and with *ced-9*, which is required to protect cells that should live (Hengartner, 1997). As expected, *ced-3(RNAi)* and *ced-4(RNAi)* F1 animals (i.e., the progeny of animals in which *ced-3* or *ced-4* was inactivated by RNAi, respectively) had extra cells, whereas ced-9(RNAi) Po animals were either sterile or only generated dead embryos (data not shown). From these experiments, we concluded that RNAi can be used to determine whether our candidate genes have any role in programmed cell death.

So far, we have been able to obtain preliminary results for five of the genes, including the three that encode potential BH3 domain proteins (Table 2). Unfortunately, none of them showed any obvious cell death phenotype, be it extra cells or extra death (Table 2). We did detect some other defects, such as embryonic lethality. However, while these phenotypes are interesting in their own right, and might tell us something about the normal function of our candidate genes, we do not plan to pursue the characterization of these clones any further under this grant, as they have nothing to do with our stated objectives.

We expect that by next year, we will have concluded our RNAi analysis of all the clones.

6.4 Progress evaluation

We are on track or even well ahead of schedule for all the tasks that we submitted with our statement of work in our original application.

1. For CED-4/Apaf-1, we are close to having completed all the experiments that we proposed to do with a bona fide CED-9 interactor, and starting to develop our project beyond the scope of this grant.
2. For the other clones, we are now in the process of determining biological function in *C. elegans*, which in our original statement of work we planned to be doing in months 14-24. We are on schedule to begin our work with the mammalian homologs of these candidates. However, we believe that such work only makes sense once the *C. elegans* data suggests that we have a bona fide cell death gene in our hands.

We plan to continue our work on the remaining 11 candidate genes according to the statement of work originally submitted, and do not foresee any particular problems in respecting the proposed time line.

7. CONCLUSIONS

We have, in the first year of funding of our grant, made considerable progress towards our goal of using *C. elegans* to identify genes that function in apoptosis. Some of the work that we generated in this research project has already been published (Spector et al., 1997), and the thought process generated by this research program has also allowed the PI to make some intellectual contributions toward the development of a molecular model for apoptosis (Hengartner, 1997; Hengartner, 1998; Hengartner, 1998). The identification of the apoptosome in C. elegans has already generated considerable research in the mammalian field (see for example Pan et al., 1998; Hu et al., 1998).

We are looking forward to two more years of funding, and can but hope that we will continue to be as successful as we have been so far.

8. REFERENCES

Adams, J. M., and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science *281*.

Anderson, T. J., Battersby, S., and Macintyre, C. C. A. (1988). Proliferative and secretory activity in human breast during natural and artificial menstrual cycles. Am. J. Pathol. *130*, 193-204.

Chinnaiyan, A. M., O'Rourke, K., Lane, B. R., and Dixit, V. M. (1997). Interaction of CED-4 with CED-3 and CED-9: A molecular framework for cell death. Science *275*, 1122-1126.

Conradt, B., and Horvitz, H. R. (1998). The *C. elegans* protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. Cell *93*, 519-529.

Driscoll, M. (1992). Molecular genetics of cell death in the nematode *Caenorhabditis elegans*. J. Neurobiol. *23*, 1327-1351.

Ellis, H. M., and Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode *C. elegans*. Cell *44*, 817-829.

Farrow, S., and Brown, R. (1996). New members of the Bcl-2 family and their protein partners. Curr. Op. Genet. Dev. *6*, 45-49.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature *391*, 806-811.

Harlow, E., and Lane, D. (1988). Antibodies. A laboratory manual. (Cold Spring Harbor: Cold Spring Harbor Laboratory Press).

Hengartner, M. O. (1997). Apoptosis: CED-4 is a stranger no more. Nature *388*, 714-715.

Hengartner, M. O. (1998). Apoptosis: Death by crowd control. Science *281*, 1298-1299.

Hengartner, M. O. (1998). Apoptosis: Death cycle and Swiss army knives. Nature *391*, 441-442.

Hengartner, M. O. (1998). The apoptosome: molecular control of apoptosis in the nematode *C. elegans*. In Pharmaceutical interventions in apoptotic pathways., J. F. Nagelkerke, J. H. van Dierendonck and M. H. M. Noteborn, eds. (Amsterdam, The Netherlands: North – Holland), pp. 45-51.

Hengartner, M. O. (1997). Cell Death. In C. elegans II, D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess, eds. (Cold Spring Harbor: Cold Spring Harbor Laboratory Press), pp. 383-415.

Hengartner, M. O., Ellis, R. E., and Horvitz, H. R. (1992). *C. elegans* gene *ced-9* protects cells from programmed cell death. Nature *356*, 494-499.

Hengartner, M. O., and Horvitz, H. R. (1994). Activation of *C. elegans* cell death protein CED-9 by an amino-acid substitution in a domain conserved in Bcl-2. Nature *369*, 318-320.

Hengartner, M. O., and Horvitz, H. R. (1994). *C. elegans* cell death gene *ced-9* encodes a functional homolog of mammalian proto-oncogene *bcl-2*. Cell *76*, 665-676.

Hengartner, M. O., and Horvitz, H. R. (1994). The ins and outs of programmed cell death in *C. elegans*. Phil. Trans. R. Soc. Lond. B *345*, 243-246.

Hockenbery, D. M., Oltvai, Z. N., Yin, X.-M., Milliman, C. L., and Korsmeyer, S. J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell *75*, 241-251.

Hoskins, K., and Weber, B. L. (1995). Recent advances in breast cancer biology. Curr. Opin. Oncol. *7*, 495-500.

Hu, Y., Benedict, M. A., Wu, D., Inohara, N., and Nunez, G. (1998). Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. Proc. Natl. Acad. Sci. USA *95*, 4386-4391.

Hunter, J. J., Bond, B. L., and Parslow, T. G. (1996). Functional dissection of the human Bcl-2 protein: sequence requirements for inhibition of apoptosis. Mol. Cell. Biol. *16*, 877-883.

James, C., Gschmeissner, S., Fraser, A., and Evan, G. I. (1997). CED-4 induces chromatin condensation in *Schizosaccharomyces pombe* and is inhibited by direct physical association with CED-9. Curr. Biol. *7*, 246-252.

Kumar, S. (1995). ICE-like proteases in apoptosis. Trends Biochem. Sci. *20*, 198-202.

Longacre, T. A., and Bartow, S. A. (1986). A correlative morphologic study of human breast and endometrium in the menstrual cycle. Am. J. Surg. Pathol. *10*, 382-393.

Lowe, S. W., Bodis, S., Bardeesy, N., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., Jacks, T., Pelletier, J., and Housman, D. E. (1994). Apoptosis and the prognostic significance of p53 mutation. Cold Spring Harb. Symp. Quant. Biol. *59*, 419-26.

Miura, M., Zhu, H., Rotello, R., Hartwieg, E. A., and Yuan, J. (1993). Induction of apoptosis in fibroblasts by IL-1ß converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced-3*. Cell *75*, 653-660.

Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Chang, B. S., Thompson, C. B., Wong, S. L., Ng, S. L., and Fesik, S. W. (1996). X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature *381*, 335-341.

Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998). An induced proximity model for caspase-8 activation. J. Biol. Chem. *273*, 2926-2930.

Pan, G., O'Rourke, K., and Dixit, V. M. (1998). Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. J. Biol. Chem. *273*, 5841-5845.

Schulte-Hermann, R., Bursch, W., Grasl-Kraupp, B., Torok, L., Ellinger, A., and Mullauer, L. (1995). Role of active cell death (apoptosis) in multi-stage carcinogenesis. Toxicol. Lett. *82-83*, 143-8.

Shaham, S., and Horvitz, H. R. (1996). An alternatively spliced *C. elegans ced-4* RNA encodes a novel cell death inhibitor. Cell *86*, 201-208.

Shaham, S., and Horvitz, H. R. (1996). Developing *Caenorhabditis elegans* neurons may contain both cell-death protective and killer activities. Genes & Dev. *10*, 578-591.

Spector, M. S., Desnoyers, S., Hoeppner, D. J., and Hengartner, M. O. (1997). Interaction between the C. elegans cell-death regulators CED-9 and CED-4. Nature *385*, 653-656.

Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E. S. (1998). Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol Cell *1*, 949-957.

Strange, R., Friis, R. R., Bemis, L. T., and Geske, F. J. (1995). Programmed cell death during mammary gland involution. Methods Cell Biol *46*, 355-68.

Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. Science *267*, 1456-1462.

Van Aelst, L., Joneson, T., and Bar-Sagi, D. (1996). Identification of a novel Rac 1-interacting protein involved in membrane ruffling. EMBO J. *15*, 3778-3786.

Vaux, D. L., Weissman, I. L., and Kim, S. K. (1992). Prevention of programmed cell death in *Caenorhabditis elegans* by human *bcl-2*. Science *258*, 1955-1957.

Walker, N. I., Bennett, R. E., and Kerr, J. F. (1989). Cell death by apoptosis during involution of the lactating breast in mice and rats. Am J Anat *185*, 19-32.

Wu, D., Wallen, H. D., and Nuñez, G. (1997). Interaction and regulation of subcellular localization of CED-4 by CED-9. Science *275*, 1126-1129.

Yang, X., Chang, H. Y., and Baltimore, D. (1998). Essential role of CED-4 oligomerization in CED-3 activation and apoptosis. Science *In press*..

Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. (1993). The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1beta converting enzyme. Cell *75*, 641-652.

Zou, H., Henzel, W. J., Liu, X., Lutschg, A., and Wang, X. (1997). Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell *90*, 405-413.

9. APPENDICES

9.1 Appendix I. Figures and Table

9.1.1 *FIGURE 1 LEGEND*

CED-9 and CED-4 interact in the yeast two-hybrid system.
LexA and $GAL4_{AD}$ constructs were co-transformed into yeast strain L40 and colonies selected on SD-medium lacking leucine and tryptophan. The interaction between the fusion proteins was detected by the induction of ß-galactosidase (dark color, assayed as described in Ref. Van Aelst,). Each patch shown represents an independent yeast transformant.

9.1.2 *FIGURE 2 LEGEND*

Identification of the CED-9 domain required for interaction with CED-4 *in vitro*.
(A, B) $CED\text{-}4_S$ and $CED\text{-}4_L$ bind to GST-CED-9. Radiolabeled full-length *ced-*4_S and *ced-*4_L ORFs were incubated with increasing amounts (two-fold in A and ten-fold in B) of immobilized GST fusion proteins produced in *E. coli*. Bound proteins were analyzed by SDS-PAGE followed by autoradiography.

(C) Interaction between CED-9 truncations and CED-4 isoforms.
ced-9 fragments fused in-frame to GST were tested for interaction with CED-4 isoforms as described in Methods. Relative strength of interaction was determined by comparing the intensities of radiolabeled bands on autoradiograms (+++, ++, +, and - correspond to the recovery of about 10%, 2-5%, 0.2-1.0%, and <0.05% of the input radiolabeled protein, respectively; the CDK4/GST-p16 positive control scored a +++ in our assay). Position of BH domains, as defined by Muchmore et al., 1996 (black boxes) and C-terminal hydrophobic tail (gray box) are indicated. *, position of point mutation in G169E and Y149N constructs.

9.1.3 *FIGURE 3 LEGEND*

The *C. elegans* apoptosome: a model for the mechanism of action of the cell death machinery.
The cell death regulators CED-3, CED-4, and CED-9 are predicted to be stably associated in a multiprotein complex localized, by analogy with Bcl-2 family members in mammals, to the outer surface of mitochondria. This complex would be expected to be present in all cells, but is inactive. In cells fated to die, The BH3 domain protein EGL-1 dislodges CED-4/CED-3 from its CED-9 anchor, leading to oligomerization of the complex and, through poorly characterized steps that might involve ATP hydrolysis, intermolecular processing of CED-3.The active protease then cleaves the relevant apoptotic substrates, bringing on the death of the cell.

9.1.4 *FIGURE 4 LEGEND*

Monoclonal antibodies to CED-9 detect a single major band in total *C. elegans* extracts
A, Epitope map. Monoclonal antibodies (MAbs) C9.1, C9.3, and C9.6 were tested for their ability to recognize a number of CED-9 truncations. The three MAbs appear to recognize distinct epitopes on the CED-9 protein.
B, Western blot. MAbs were used to probe western blots of either wild-type (strain N2) or *ced-9(n2812)* mixed stage whole worm extracts. All three MAbs recognize a single major band in the wild type, which is absent in the *ced-9* null mutant. Western blotting was performed using standard protocols (Harlow and Lane, 1988).

FIGURE 1

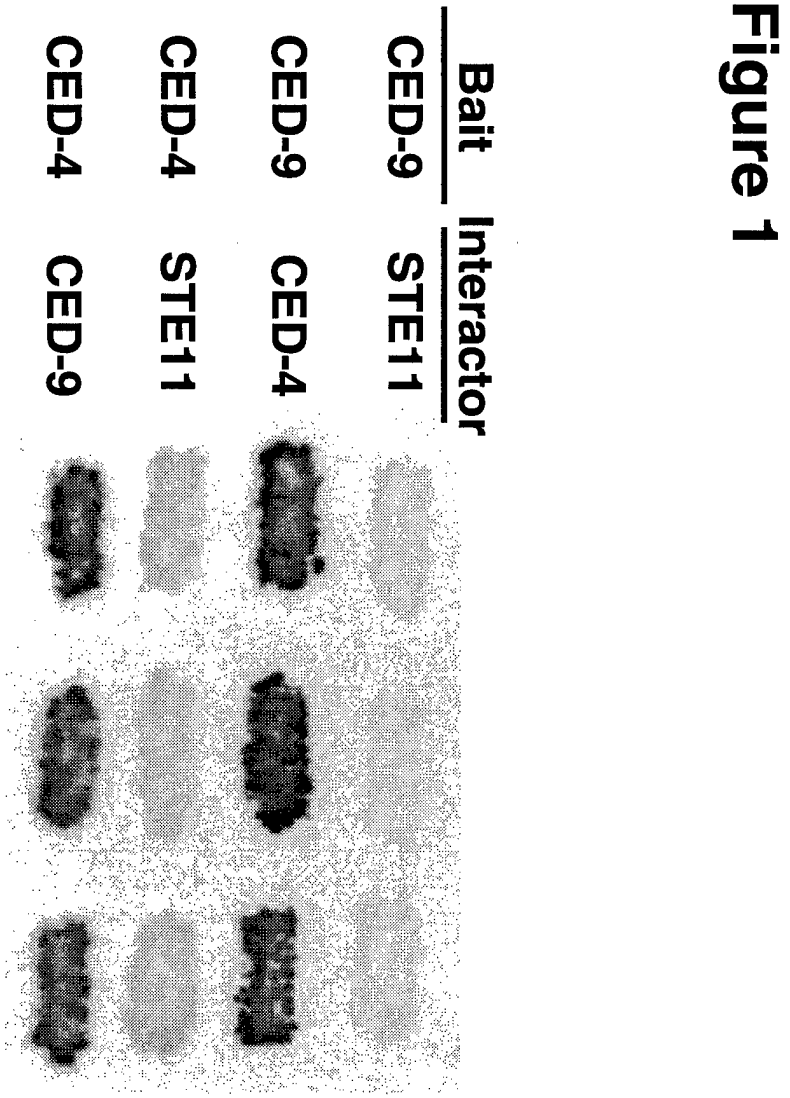
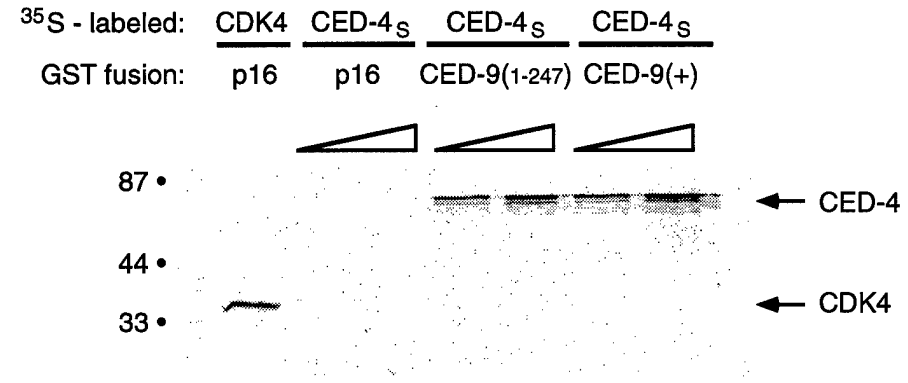


Figure 1
Bait
Interactor
CED-9
STE11
CED-9
CED-4
CED-4
STE11
CED-4
CED-9

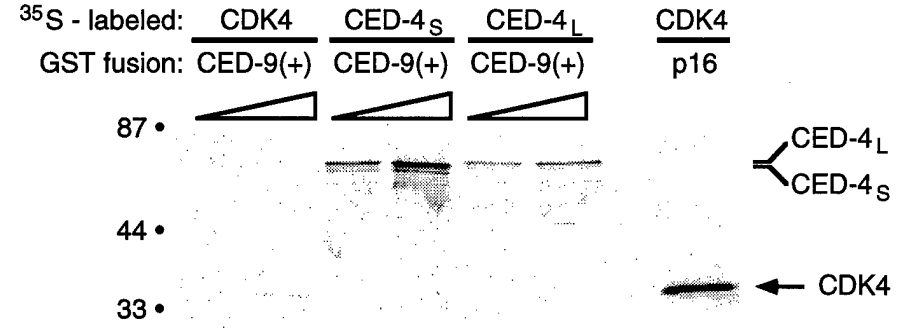
FIGURE 2

Figure 2

A



B



C

CED-9 construct	BH3	BH1	BH2	Tail	Interaction with CED-4S	Interaction with CED-4L
1-280					++	+
G169E		*			++	+
Y149N		*			+	+
1-247					++	+
1-231					+	+
1-159					-	-
97-280					++	+
97-231					+	+

FIGURE 3

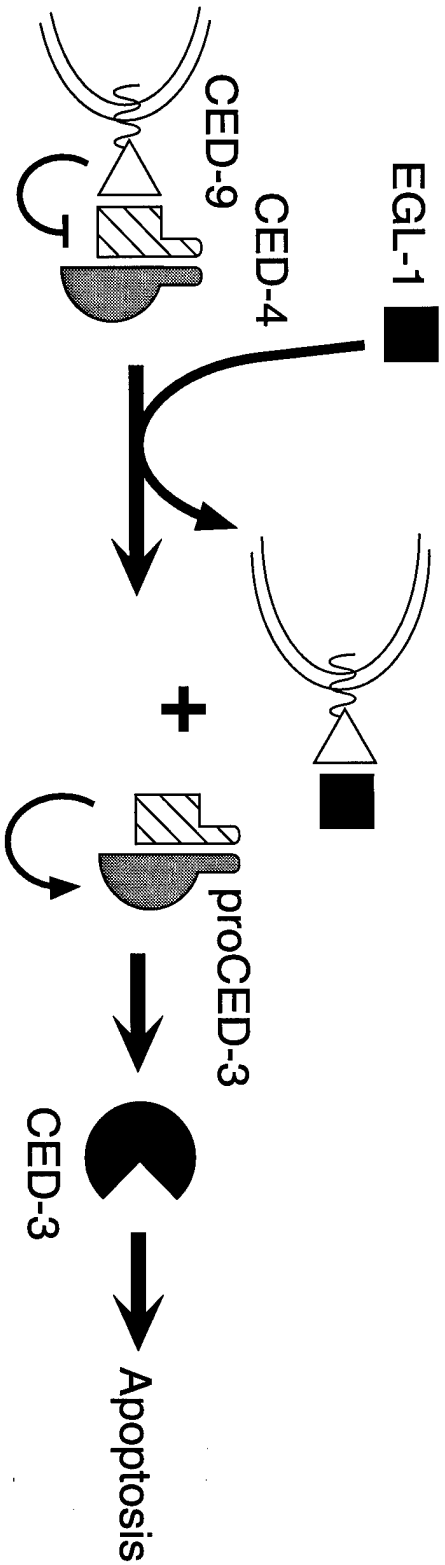
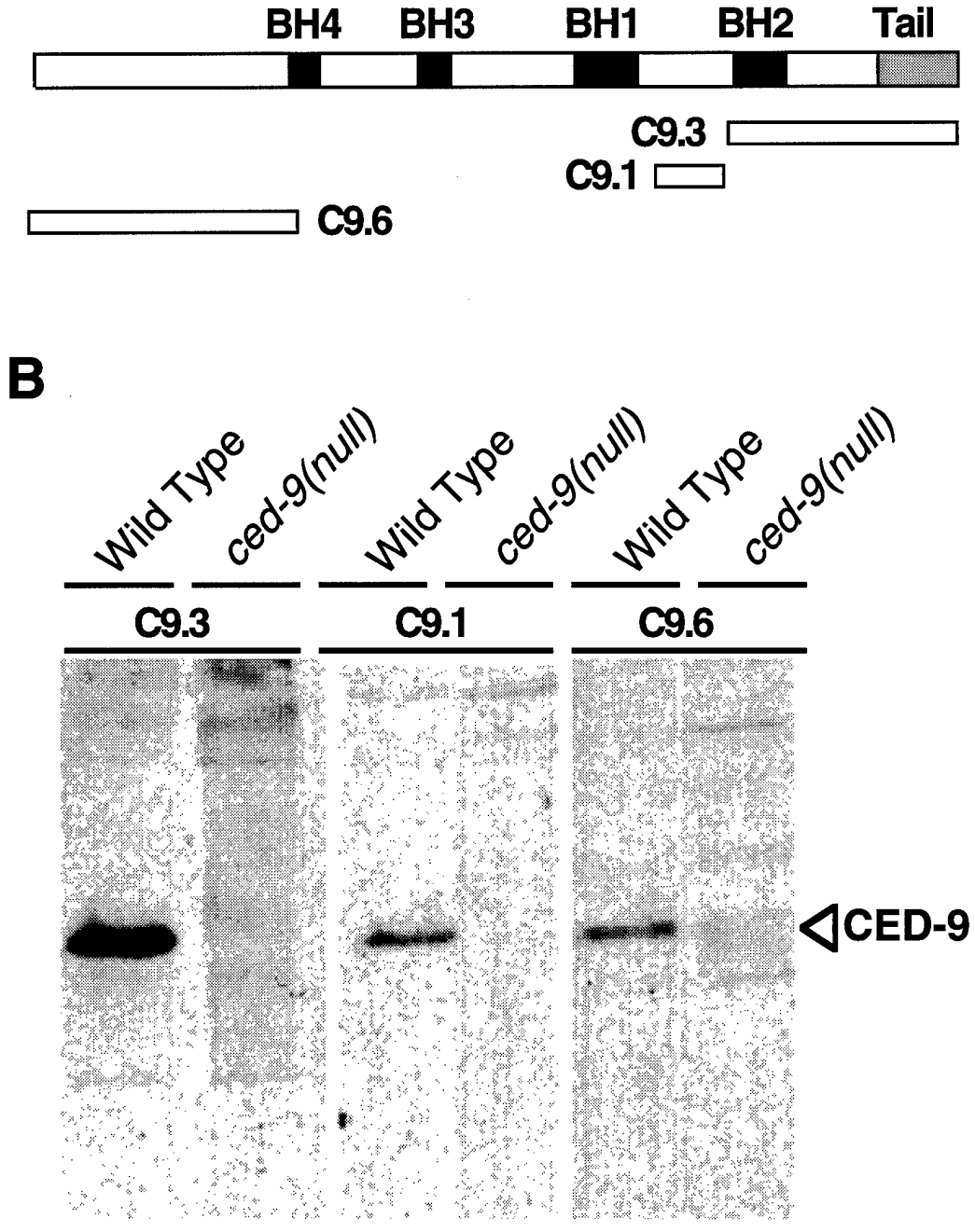


Figure 3

FIGURE 4

Figure 4



9.1.5 *TABLE 1*

Mutational analysis of the CED-9 / CED-4 interaction in the yeast two-hybrid system.

Bait (LexA fusion)	Interactor ($GAL4_{AD}$ fusion)	Color Intensity (filters)	Relative ß-galactosidase activity
STE11	STE11	+++	4100 ± 400
$CED\text{-}4_S$	CED-9	+++	18.7 ± 1.5
$CED\text{-}4_S$	STE11	+/-	3.6 ± 1.4
CED-9	$CED\text{-}4_S$	+++	541 ± 29
CED-9	STE11	-	1.0 ± 0.3
$CED\text{-}4_S$	CED-9(1-231)	+++	1080 ± 320
STE11	CED-9(1-231)	-	0.6 ± 0.1
$CED\text{-}4_S$	CED-9(G169E) (*gf*)	+++	42.4 ± 4.2
CED-9(G169E) (*gf*)	$CED\text{-}4_S$	+++	455 ± 93
CED-9(G169E) (*gf*)	STE11	-	0.7 ± 0.2
STE11	CED-9(G169E) (*gf*)	-	0.9 ± 0.1
$CED\text{-}4_S$	CED-9(Y149N) (*lf*)	-	0.9 ± 0.3
$CED\text{-}4_S$	CED-9(1-159) (*lf*)	-	0.6 ± 0.2
STE11	CED-9(Y149N) (*lf*)	-	0.5 ± 0.1
STE11	CED-9(1-159) (*lf*)	-	0.3 ± 0.1

Interaction strength was determined both on filters and in liquid Van Aelst, In press; Van Aelst et al., 1996. Intensity of the blue color on filters was assessed on a qualitative scale, from - (white) to +++ (as strong as the LexA-STE11 / $GAL4_{AD}$-STE11 positive control). ß-galactosidase activities shown correspond to average ± s.e.m. of three independent transformants assayed in duplicate, and are shown as relative activities compared to the negative control (LexA-CED-9 / $GAL4_{AD}$-STE11). All fusion proteins were stably expressed, as determined by western blotting (data not shown). gf, gain-of-function; lf, loss-of-function.

9.1.6 *TABLE 2*

LexA-CED-9 interacting clones

Clone(s)	Gene	LG	RNAi phenotype	Function/Homology
A/T3/H	C05D11.7	III	embryonic lethality	GS2
I/L	B0303.4	III	ND	none
J/P	F56C9.7	III	ND	cation transporter
O	T07C4.3	III	DTC migration defect	none
T4	C15B12.7	X	ND	cobalt uptake
C	Y47E8	V	embryonic lethality	SEC23
E	F52B5.1	I	none	anion exchange
F	T22B11.4	IV	ND	none
Q	W06A7.3a	V	ND	none
M	H21P03.a	IV	ND	none
T2	T02D7	I	extra pharyngeal cell	*unc-40*

Clones were sequenced at both ends and mapped to cosmids and/or YACs by comparing the obtained sequence with the known *C. elegans* genomic sequence. LG, linkage group (chromosome). ND, not determined. Genes were deemed to be homologous if they showed a P value of less that 10E-05 on a blast search (using the NCBI blast server).

Appendix II. Selected Reprints

1. Spector, M. S., Desnoyers, S., Hoeppner, D. J., and Hengartner, M. O. (1997). Interaction between the C. elegans cell-death regulators CED-9 and CED-4. Nature *385*, 653-656.

2. Hengartner, M. O. (1997). Apoptosis: CED-4 is a stranger no more. Nature *388*, 714-715.

3. Hengartner, M. O. (1998). Apoptosis: Death cycle and Swiss army knives. Nature *391*, 441-442.

Interaction between the *C. elegans* cell-death regulators CED-9 and CED-4

Mona S. Spector*, Serge Desnoyers*, Daniel J. Hoeppner*† & Michael O. Hengartner*

* *Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA*
† *Graduate Program in Genetics, Department of Molecular Microbiology, SUNY at Stony Brook, Stony Brook, New York 11794, USA*

Programmed cell death (apoptosis) is an evolutionarily conserved process used by multicellular organisms to eliminate cells that are not needed or are potentially detrimental to the organism[1,2]. Members of the Bcl-2 family of mammalian proteins are intimately involved in the regulation of apoptosis, but, their precise mechanism of action remains unresolved[3–5]. In *Caenorhabditis elegans*, the Bcl-2 homologue CED-9 prevents cell death by antagonizing the death-promoting activities of CED-3, a

member of the Caspase family of death proteases, and of CED-4, a protein with no known mammalian homologue[6–9]. Here we show that CED-9 interacts physically with CED-4. Mutations that reduce or eliminate CED-9 activity also disrupt its ability to bind CED-4, suggesting that this interaction is important for CED-9 function. Thus, CED-9 might control *C. elegans* cell death by binding to and regulating CED-4 activity. We propose that mammalian Bcl-2 family members might control apoptosis in a similar way through interaction and regulation of CED-4 homologues or analogues.

Genetic analysis of programmed cell death in the nematode *C. elegans* has led to the identification of over a dozen cell-death (*ced*) genes[10,11]. Two of these genes, *ced-3* and *ced-4*, are required for cells to die: in animals carrying a mutation in either gene, all programmed cell death is blocked[12]. The *ced-3* gene encodes a homologue of the mammalian Caspase family of death proteases[9,13]. *ced-4* is predicted to encode, through alternative splicing, two related but distinct proteins: the major, shorter isoform (CED-4_S) promotes apoptosis[8], whereas a less abundant, longer isoform (CED-4_L) antagonizes cell death[14]. Neither isoform shares any strong sequence similarity with previously characterized proteins[8,11,15]. A third *C. elegans* gene, *ced-9*, protects *C. elegans* cells from apoptotic death[6,7,16]. Overexpression of *ced-9* results in the survival of cells that should die; a similar phenotype is also observed in animals carrying the dominant gain-of-function mutation *ced-9(n1950)*. In contrast, *ced-9* loss-of-function mutations result in the widespread death of cells that would normally survive. The CED-9 protein is a member of the Bcl-2 family of cell-death regulators, and Bcl-2 can prevent cell death in *C. elegans* by partially substituting for CED-9 (refs 7,17). The conservation in sequence and function of the CED-3/Caspase and CED-9/Bcl-2 protein families suggests that *C. elegans* and mammals use a similar molecular program to regulate apoptosis[5,10].

In *C. elegans*, genetic studies indicate that CED-9 prevents cell death by antagonizing the death-promoting activities of CED-3 and CED-4 (refs 6, 7, 16, 18). To determine whether this suppressive activity might be mediated through a direct interaction, we investigated whether these proteins interact in the yeast two-hybrid system[19]. We found that full-length CED-9 binds strongly to CED-4_S, the death-promoting form of CED-4 (Fig. 1 and Table 1). This interaction, which could be detected using either CED-9 or CED-4_S as the bait, is specific, as neither LexA–CED-9 nor LexA–CED-4_S interact with other proteins used as negative controls ($GAL4_{AD}$–STE11 and $GAL4_{AD}$–Lamin; Fig. 1 and Table 1, and data not shown).

To determine whether this CED-9/CED-4 interaction in our yeast two-hybrid system was direct, we tested CED-9 and CED-4 for interaction *in vitro*. We found that *in vitro*-translated CED-4_S protein readily bound to a glutathione-*S*-transferase–CED-9 (GST–CED-9) fusion protein (Fig. 2a, b); there was little binding of CED-4_S and GST–CED-9 to GST–p16 and CDK4 (two irrelevant proteins used as negative controls), respectively, confirming that the CED-9/CED-4_S interaction is specific (Fig. 2a, b).

Given the strong genetic link between *ced-9* and *ced-4*, it is likely that the physical interaction between CED-9 and CED-4 is biologically important. We therefore looked for a correlation between the ability of CED-9 to interact with CED-4 and its capacity to prevent cell death. We introduced point mutations into the *ced-9* open reading frame to recreate three CED-9-mutant proteins whose effect on cell death we had previously characterized: CED-9(G169E), the product of the dominant gain-of-function allele *ced-9(n1950)*; CED-9(Y149N), which corresponds to the loss-of-function mutation *ced-9(n1653*ts); and Q160stop, which results in the truncated protein CED-9(1–159) and corresponds to the loss-of-function mutation *ced-9(n2077)* (refs 6, 7, 16).

The *n1950* gain-of-function mutation results in a glycine-to-glutamate (G169E) substitution in the conserved Bcl-2 homology (BH) domain BH1, which is present in and mediates interactions between most Bcl-2 family members[20]. Consistent with the observation that *n1950* does not eliminate *ced-9* function, we found that CED-9(G169E) interacts with CED-4_S (Table 1 and Fig. 2c). Because the CED-9(G169E)/CED-4_S interaction was not significantly stronger than the one we observed between CED-9(+) and CED-4_S, *n1950* is unlikely to cause a gain of function by simply increasing CED-9/CED-4 interaction. Rather, we suspect—on the basis of the observation that human Bcl-2 can interact with many distinct proteins—that *n1950* affects the ability of CED-9 to interact with another, as-yet unidentified partner, possibly the nematode equivalent of Bax. Alternatively, *n1950* might alter the *in vivo* regulation of the CED-9/CED-4 interaction in ways that cannot be detected in our assay systems.

In contrast, the Y149N and Q160stop loss-of-function mutations both abrogated interaction between CED-9 and CED-4 in the yeast

Table 1 Mutational analysis of the CED-9/CED-4 interaction in the yeast two-hybrid system

Bait (LexA fusion)	Interactor ($GAL4_{AD}$ fusion)	Colour intensity (filters)	Relative β-galactosidase activity
STE11	STE11	+++	4,100 ± 400
CED-4_S	CED-9	+++	18.7 ± 1.5
CED-4_S	STE11	+/−	3.6 ± 1.4
CED-9	CED-4_S	+++	541 ± 29
CED-9	STE11	−	1.0 ± 0.3
CED-4_S	CED-9(1-231)	+++	1,080 ± 320
STE11	CED-9(1-231)	−	0.6 ± 0.1
CED-4_S	CED-9(G169E) (*gf*)	+++	42.4 ± 4.2
CED-9(G169E) (*gf*)	CED-4_S	+++	455 ± 93
CED-9(G169E) (*gf*)	STE11	−	0.8 ± 0.1
STE11	CED-9(G169E) (*gf*)	−	0.9 ± 0.1
CED-4_S	CED-9(Y149N) (*lf*)	−	0.9 ± 0.3
CED-4_S	CED-9(1-159) (*lf*)	−	0.6 ± 0.2
STE11	CED-9(Y149N) (*lf*)	−	0.5 ± 0.1
STE11	CED-9(1-159) (*lf*)	−	0.3 ± 0.1

Interaction strength was determined both on filters and in liquid[26,27]. Intensity of the blue colour on filters was assessed on a qualitative scale, from '−' (white) to '+++' (as strong as the LexA-STE11/$GAL4_{AD}$-STE11 positive control). β-Galactosidase activities shown correspond to average ± s.e.m. of three independent transformants assayed in duplicate, and are shown as relative activities compared to the negative control (LexA-CED-9/$GAL4_{AD}$-STE11). All fusion proteins were stably expressed, as determined by western blotting (data not shown). *gf*, Gain-of-function; *lf*, loss-of-function.

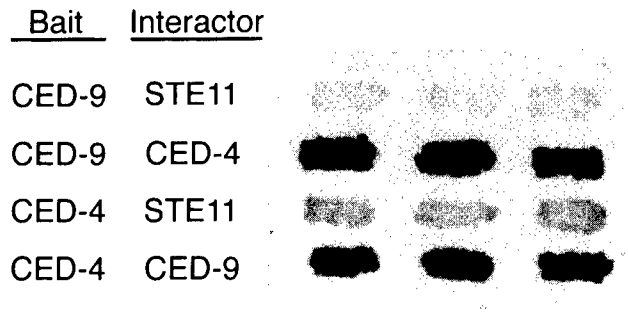


Figure 1 CED-9 and CED-4 interact in the yeast two-hybrid system. LexA and $GAL4_{AD}$ constructs were co-transformed into yeast strain L40 and colonies selected on SD medium lacking leucine and tryptophan. The interaction between the fusion proteins was detected by the induction of β-galactosidase (dark colour, assayed according to ref. 27). Each patch represents an independent yeast transformant.

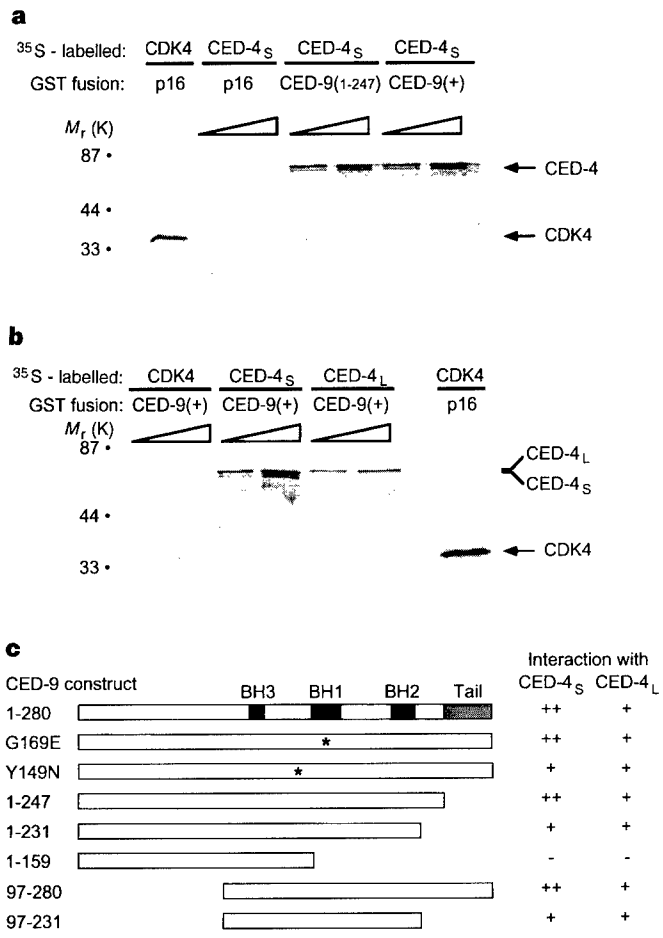


Figure 2 Identification of the CED-9 domain required for interaction with CED-4 *in vitro*. **a**, **b**, CED-4_S and CED-4_L bind to GST-CED-9. Radiolabelled full-length *ced-4*$_S$ and *ced-4*$_L$ ORFs were incubated with increasing amounts (twofold in **a** and tenfold in **b**) or immobilized GST fusion proteins produced in *E. coli*. Bound proteins were analysed by SDS-PAGE, followed by autoradiography. **c**, Interaction between CED-9 truncations and CED-4 isoforms. *ced-9* fragments fused in-frame to GST were tested for interaction with CED-4 isoforms as described in Methods. Relative strength of interaction was determined by comparing the intensities of radiolabelled bands on autoradiograms (+++, ++, +, and − correspond to the recovery of about 10, 2–5, 0.2–1.0 and <0.05% of the input radiolabelled protein, respectively; the CDK4/GST-p16 positive control scored a +++ in our assay). The positions of the BH domains[29] (black boxes) and C-terminal hydrophobic tail (grey box) are indicated. Asterisk, position of the point mutation in G169E and Y149N constructs.

two-hybrid system (Table 1). Q160stop also eliminated the CED-9/CED-4 interaction *in vitro*, whereas Y149N only reduced it (Fig. 2c). The weak effect of Y149N on CED-9/CED-4 interaction *in vitro* is consistent with the temperature-sensitive nature of this mutation[6], and the different temperatures at which our assays were performed (30 °C for yeast; 4 °C *in vitro*). *In vivo*, Y149N might prevent CED-9/CED-4 interaction only at high but not at low temperatures (we have excluded trivial explanations, such as reduced protein stability for the *n1653* temperature sensitivity, as the Y149N protein is as stable as the wild-type protein under both permissive and restrictive temperatures; M.S.S., unpublished results). The correlation between loss of *ced-9* function and loss of CED-4 binding is consistent with the hypothesis that direct interaction with CED-4 is important for CED-9 to prevent cell death.

To identify the domain(s) of CED-9 required for interaction with CED-4, we generated several CED-9-deletion constructs and tested their ability to interact with full-length CED-4_S *in vitro* (Fig. 2c). Several Bcl-2 family members contain a C-terminal hydrophobic tail, which drives these proteins to the outer surface of the mitochondrion, the rough endoplasmic reticulum and nuclear membranes[21–23]. Truncations that remove this tail only slightly reduce the ability of Bcl-2 to prevent apoptosis[24,25]. Similarly, we found that CED-9(1–247), which lacks the hydrophobic tail, still prevents programmed cell death when overexpressed in *C. elegans* (data not shown). Thus, the C-terminal tail is not essential for Bcl-2 or CED-9 function. Consistent with these observations, CED-9(1–231), which lacks the C-terminal 49 amino acids, still interacted with CED-4_S (Table 1 and Fig. 2c). However, elimination of a further 72 amino acids, which include the BH1 and BH2 domains (CED-9(1–159) versus CED-9(1–231)), completely prevented CED-9 from interacting with CED-4_S both *in vitro* and in yeast (Table 1 and Fig. 2c), indicating that the BH1 and BH2 domains, which are important for the function of several Bcl-2 family members, might be involved in CED-9/CED-4 interaction. In support of this, residues 97–231 (containing the BH1, BH2 and BH3 domains) were sufficient for interaction with CED-4_S (Fig. 2c).

An alternative splice variant of *ced-4*, *ced-4*$_L$, has been described[14]. This variant encodes a putative protein containing 24 additional amino acids. In contrast to the principal isoform (*ced-4*$_S$), overexpression of *ced-4*$_L$ prevents death. However, both forms of *ced-4* are antagonized by *ced-9* (ref. 14), possibly explaining why *ced-9* also has a minor death-promoting activity[16]. Because *ced-9* antagonizes both *ced-4*$_L$ and *ced-4*$_S$, we suspected that CED-9 could also interact with CED-4_L. Indeed, we found no qualitative differences between CED-4_S and CED-4_L in their ability to bind to CED-9, although the CED-9/CED-4_L interaction was invariably weaker (Fig. 2b, c). Thus, the difference between CED-4_S and CED-4_L may be in their ability to interact with or activate downstream targets, rather than in their binding to CED-9.

We have demonstrated that the *C. elegans* death suppressor CED-9 interacts directly with one of its downstream targets, CED-4. This interaction is disrupted in two *ced-9(lf)* mutants and might be mediated through the conserved BH1, BH2 and BH3 domains. Although our data indicate that binding to CED-4 is important for CED-9 function, we do not know whether it is sufficient. In a simple model consistent with our observations and with the genetic data, CED-9-bound CED-4_S would be unable to promote apoptosis and death-promoting stimuli would induce dissociation of the two proteins. The conservation of the death machinery between nematodes and mammals indicates that, as for *ced-3* and *ced-9*, mammals should contain one or several proteins that play a role similar to that of *ced-4* in *C. elegans*. If the CED-4-binding domain in CED-9 is conserved through evolution, then this information could be used to identify mammalian CED-4 homologues (or analogues) through their ability to interact with the equivalent domain in Bcl-2 family members. □

Methods

Site-directed mutagenesis and deletion constructs. The *n1653* mutation was introduced into a *ced-9* open reading frame (ORF) construct by site-directed mutagenesis (Quik Change, Stratagene), as recommended by the manufacturer. The *n1950* ORF was generated as described[16]. All deletion constructs were generated by polymerase chain reaction (PCR) using appropriate primers. To obtain a full-length *ced-4*$_L$ clone, PCR was used with reverse transcription to amplify (from total, mixed-stage RNA) a cDNA fragment covering the alternatively spliced exon[14]; this *ced-4*$_L$ fragment was then used to replace the corresponding region in *ced-4*$_S$ using unique restriction sites present in the *ced-4* ORF. The DNA sequence of all constructs used were confirmed by fluorescent sequencing on an ABI 377 Sequencer.

Yeast two-hybrid assay. *ced-9* and *ced-4* cDNAs and fragments were cloned in-frame into the yeast two-hybrid vectors pBTM116 (to create LexA fusions) and pGAD-GH (to create $GAL4_{AD}$ fusions)[26,27]. LexA and $GAL4_{AD}$ constructs were co-transformed into yeast strain L40 and colonies selected on SD medium

lacking leucine and tryptophan. LexA–STE11, $GAL4_{AD}$–STE11, and $GAL4_{AD}$–lamin constructs used in control experiments were generously provided by L. Van Aelst. β-Galactosidase was assayed as described[28].

***In vitro* interaction.** The *ced-9* open reading frame and various deletion fragments were subcloned in-frame into the GST fusion construct pGEX-1λT (Pharmacia). GST fusion constructs were transformed into *Escherichia coli* strain BL21(DE3) and induced by addition of IPTG. Fusion proteins were purified using glutathione–Sepharose beads (Pharmacia), as suggested by the manufacturer. Full-length *ced-*4_S and *ced-*4_L ORFs in pCITE (Novagen) were used as templates in an *in vitro* transcription/translation reaction (TnT, Promega) in the presence of ^{35}S-methionine. Pre-cleared *in vitro* transcription/translation lysates were added to 200 μl incubation buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 2 mM EDTA, 5 mM DTT, 0.5% Nonidet P-40) containing the GST fusion protein (between 0.2 and 2.0 μg, depending on the experiment) immobilized on glutathione–Sepharose, incubated for 1 h at 4 °C, and washed four times with incubation buffer. Bound proteins were analysed by SDS–PAGE followed by autoradiography.

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1. Ellis, R. E., Yuan, J. & Horvitz, H. R. Mechanisms and functions of cell death. *Annu. Rev. Cell Biol.* **7,** 663–698 (1991).
2. Thompson, C. B. Apoptosis in the pathogenesis and treatment of disease. *Science* **267,** 1456–1462 (1995).
3. Hacker, G. & Vaux, D. L. A sticky business. *Curr. Biol.* **5,** 622–624 (1995).
4. Korsmeyer, S. J. Regulators of cell death. *Trends Genet.* **11,** 101–105 (1995).
5. Chinnaiyan, A. M. & Dixit, V. M. The cell-death machine. *Curr. Biol.* **6,** 555–562 (1996).
6. Hengartner, M. O., Ellis, R. E. & Horvitz, H. R. *C. elegans* gene *ced-9* protects cells from programmed cell death. *Nature* **356,** 494–499 (1992).
7. Hengartner, M. O. & Horvitz, H. R. *C. elegans* cell death gene *ced-9* encodes a functional homolog of mammalian proto-oncogene *bcl-2. Cell* **76,** 665-676 (1994).
8. Yuan, J. & Horvitz, H. R. The *Caenorhabditis elegans* cell death gene *ced-4* encodes a novel protein and is expressed during the period of extensive programmed cell death. *Development* **116,** 309–320 (1992).
9. Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M. & Horvitz, H. R. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1β converting enzyme. *Cell* **75,** 641–652 (1993).
10. Hengartner, M. O. & Horvitz, H. R. Programmed cell death in *C. elegans. Curr. Opin. Gen. Dev.* **4,** 581–586 (1994).
11. Horvitz, H. R., Shaham, S. & Hengartner, M. O. The genetics of programmed cell death in the nematode *Caenorhabditis elegans. Cold Spring Harbor Symp. Quant. Biol.* **LIX,** 377–385 (1994).
12. Ellis, H. M. & Horvitz, H. R. Genetic control of programmed cell death in the nematode *C. elegans. Cell* **44,** 817–829 (1986).
13. Alnemri, E. S. *et al.* Human ICE/CED-3 protease nomenclature. *Cell* **87,** 171 (1996).
14. Shaham, S. & Horvitz, H. R. An alternatively spliced *C. elegans ced-4* RNA encodes a novel cell death inhibitor. *Cell* **86,** 201–208 (1996).
15. Nagata, S. Telling cells their time is up. *Curr. Biol.* **6,** 1241–1243 (1996).
16. Hengartner, M. O. & Horvitz, H. R. Activation of *C. elegans* cell death protein CED-9 by an amino-acid substitution in a domain conserved in Bcl-2. *Nature* **369,** 318–320 (1994).
17. Vaux, D. L., Weissman, I. L. & Kim, S. K. Prevention of programmed cell death in *Caenorhabditis elegans* by human *bcl-2. Science* **258,** 1955–1957 (1992).
18. Shaham, S. & Horvitz, H. R. Developing *Caenorhabditis elegans* neurons may contain both cell-death protective and killer activities. *Genes Dev.* **10,** 578–591 (1996).
19. Chien, C. T., Bartel, P. L., Sternglanz, R. & Fields, S. The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc. Natl Acad. Sci. USA* **88,** 9578–9582 (1991).
20. Farrow, S. & Brown, R. New members of the Bcl-2 family and their protein partners. *Curr. Opin. Genet. Dev.* **6,** 45–49 (1996).
21. Chen-Levy, Z., Nourse, J. & Cleary, M. L. The bcl-2 candidate proto-oncogene product is a 24-kilodalton integral-membrane protein highly expressed in lymphoid cell lines and lymphomas carrying the t(14;18) translocation. *Mol. Cell. Biol.* **9,** 701–710 (1989).
22. Hockenbery, D., Nuñez, G., Milliman, C., Schreiber, R. D. & Korsmeyer, S. J. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* **348,** 334–336 (1990).
23. Krajewski, S. *et al.* Immunohistochemical determination of *in vivo* distribution of Bax, a dominant inhibitor of Bcl-2. *Am. J. Pathol.* **145,** 1323–1336 (1994).
24. Hockenbery, D. M., Oltvai, Z. N., Yin, X.-M., Milliman, C. L. & Korsmeyer, S. J. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75,** 241–251 (1993).
25. Hunter, J. J., Bond, B. L. & Parslow, T. G. Functional dissection of the human Bcl-2 protein: sequence requirements for inhibition of apoptosis. *Mol. Cell. Biol.* **16,** 877–883 (1996).
26. Van Aelst, L., Joneson, T. & Bar-Sagi, D. Identification of a novel Rac 1-interacting protein involved in membrane ruffling. *EMBO J.* **15,** 3778–3786 (1996).
27. Van Aelst, L. Two hybrid analysis of Ras–Raf interactions. In *Methods in Molecular Biology* (Humana, Totowa, NJ, USA, in the press).
28. Miller, J. H. Assay of β-galactosidase. In *Experiments in Molecular Genetics* 352–355 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1972).
29. Muchmore, S. W. *et al.* X-ray and NMR structure of human Bcl-x_L, an inhibitor of programmed cell death. *Nature* **381,** 335–341 (1996).

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Correspondence and requests for materials should be addressed to M.O.H. (e-mail: hengartn@cshl.org).

running costs than fuel), or interfere with maintenance, either compromising safety or increasing costs.

For these reasons, riblets have not yet been used for commercial aircraft; and the new method would probably be no more attractive. However, it may have advantages for other engineering applications, for example high-speed sailing yachts, such as America's Cup competitors, for which skin friction contributes more than half the total drag. □

Peter Carpenter is in the Department of Engineering, University of Warwick, Coventry CV4 7AL, UK.

1. Walsh, M. J. *Am. Inst. Aeronautics and Astronautics Paper 82-0169* (1982).
2. Bechert, D. W., Hoppe, G. & Reif, W.-E. *Am. Inst. Aeronautics and Astronautics Paper 85-0546* (1985).
3. Sirovich, L. & Karlsson, S. *Nature* **388**, 753–755 (1997).
4. Kline, S. J., Reynolds, W. C., Schraub, F. A. & Runstadler, P. W. *J. Fluid Mech.* **95**, 741–773 (1967).
5. Smith, C. R., Walker, J. D. A., Haidari, A. H. & Sobrun, U. *Phil. Trans. R. Soc. Lond. A* **336**, 131–175 (1991).

Apoptosis

CED-4 is a stranger no more

Michael O. Hengartner

The nematode worm *Caenorhabditis elegans* has been used with great success to identify the basic components of the machinery underlying apoptosis (programmed cell death)[1]. Indeed, of the three key cell death genes that have been identified in *C. elegans*, two — *ced-3* and *ced-9* — have mammalian homologues that also function in apoptosis. But the sequence of the third gene, *ced-4*, revealed no obvious mammalian homologue, and precious little in terms of possible mechanism of action. A flurry of activity has changed that. A paper by Zou *et al.*, published earlier this month in *Cell*[2], provides a homologue. And work by Chinnaiyan *et al.* (page 728 of this issue[3]) and by Seshagiri and Miller in *Current Biology*[4] lays down some choreography for the part that CED-4 protein plays in the molecular dance of death.

Over the years, genetic screens in *C. elegans* have led to the identification of about a dozen cell death (*ced*) genes that are responsible for one aspect or another of the apoptotic process. Three of these genes stand out. Two, *ced-3* and *ced-4*, are essential for cell death. The third, *ced-9*, antagonizes the proapoptotic activities of *ced-3* and *ced-4*, and thereby protects cells that should survive from any accidental activation of the death programme.

Real stardom for *ced-9* and *ced-3* only came with their cloning, when it became clear that they encode components of a universal and highly conserved death machinery: CED-9 protein turned out to be a member of the Bcl-2 family of cell death regulators, whereas CED-3 was homologous to a family of proapoptotic cysteine proteases, known as the caspases. Indeed, identification of CED-3 as a caspase homologue was the first evidence of this family's involvement in apoptosis. In both worm and human, CED-9/Bcl-2 act upstream of the 'execution caspases' such as CED-3 and caspase-3, somehow preventing their proteolytic processing from zymogens into fully active killer enzymes. But how Bcl-2 *et al.* perform this feat remains very much up in the air.

The *ced-4* story was initially much less glamorous. Because the gene encoded a previously unknown protein without any mammalian homologue, its cloning did not provide much help in explaining how it might promote cell death. Genetically, *ced-4* had been placed between *ced-9* and *ced-3* in the pathway leading to cell death[5], suggesting that it might act as an adaptor, linking the upstream regulator CED-9 to the downstream death effector CED-3.

Strong experimental support for this view came from four papers published early this year, which collectively showed that CED-4 can interact directly and simultaneously with both CED-9 and CED-3 (reviewed in refs 6, 7); the interaction must be quite stable, as CED-4 and CED-9 co-localize when co-expressed. These observations point to a model in which CED-9 prevents cell death by directly binding to a CED-4/CED-3 complex, presumably keeping it in an inactive conformation. Upon reception of a death-inducing stimulus, the complex (already dubbed the 'apoptosome' by pundits) might dissociate, freeing CED-4/CED-3 to get to work (Fig. 1a).

But being a linker between CED-9 and CED-3 cannot be the whole story: the fact that CED-4 is essential for cell death in *C. elegans* indicates that it must have a specific proapoptotic activity. The two papers by Chinnaiyan *et al.*[3] and Seshagiri and Miller[4] describe just such an activity: both groups found that processing of proCED-3 into the active protease was dramatically increased in cells that also expressed CED-4. This stimulation was inhibited by mutations known to knock out the death-promoting activity of CED-4, or by co-expression of wild-type (but not mutant) CED-9. These observations fit very nicely with the apoptosome theory, and also explain why CED-3-mediated killing is so inefficient in the absence of CED-4.

How does CED-4 promote CED-3 activation? One possibility is that CED-4 is itself a protease that can cleave proCED-3. Arguing against this hypothesis, Chinnaiyan *et al.*[3] showed that processing of an enzymatically inactive CED-3 mutant is not stimulated by CED-4. Thus, the obvious alternative is that CED-4 promotes CED-3 autoprocessing. A hint of how it might do so comes from the observation that CED-4 contains a predicted nucleotide binding site, which seems to be functional because point mutations in the site abolish the proapoptotic activity of CED-4 in a number of experimental systems[3,4,8].

Furthermore, Chinnaiyan *et al.*[3] have now shown that purified wild-type CED-4

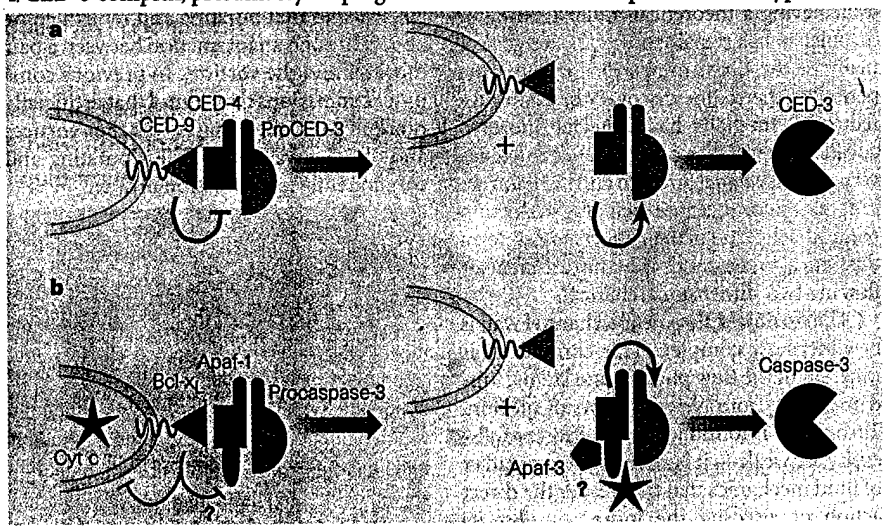

Figure 1 **Caspases are cysteine proteases that promote apoptosis in mammals and have homologues in *C. elegans*.** a and b depict highly simplified schemes of how they might be activated in the two systems. **a**, In *C. elegans* the inactive CED-9/4/3 complex is bound to cell membranes by CED-9's hydrophobic tail. On reception of a death-inducing stimulus, something happens (here shown as the physical separation of CED-4/3 from CED-9) which allows processing and activation of the 'execution caspase' CED-3. **b**, In mammals, the Bcl-2 family might perform double duty, preventing cytochrome *c* from leaving mitochondria and also possibly binding to Apaf-1 (one of the so-called apoptosis protease-activating factors, of which cytochrome *c* is Apaf-2). The death-inducing stimulus would allow formation of the full Apaf complex, possibly including Apaf-3, leading to caspase-3 processing and activation. Question marks point to aspects that are even more egregiously speculative than the rest of the model.

(but not a deletion mutant lacking the ATP binding site) can specifically bind to ATP analogues. Although nobody has yet shown that ATP hydrolysis occurs, it seems likely that CED-4 acts as a context-dependent ATPase. By analogy with the heat shock proteins, it could be that CED-4 is a proCED-3-specific chaperone, stabilizing and regulating the inactive proform. ATP hydrolysis, which might normally be inhibited by CED-9 binding, could provide the energy necessary for a conformational change and autocatalytic activation of CED-3.

Is any of this information relevant to caspase activation in mammals? The first hint that it is came from the observation by Chinnaiyan *et al.*[9] that Bcl-x_L will readily associate with caspase-1 when the two proteins are co-expressed in mammalian cells, even though they show no affinity for each other *in vitro*. The authors suggested that an endogenous, CED-4-like activity (and thus a mammalian apoptosome) exists in mammals that could act as a bridge between Bcl-x_L and caspase-1.

Even more dramatic evidence for a worm–mammal connection has been unearthed by Zou *et al.*[2]. This group had previously[10] developed an extract system derived from human cells, that, upon the addition of deoxyribose-ATP, would result in the proteolytic activation of caspase-3. Purification of the components in the extract required for this activity led to the isolation of three apoptosis protease-activating factors (Apafs), which in combination seem to be sufficient to promote caspase-3 processing upon addition of deoxyribose-ATP. So, at least functionally, the Apafs are the mammalian equivalent of *C. elegans* CED-4. Rather surprisingly, Apaf-2 is cytochrome *c*, the ubiquitous protein involved in the mitochondrial electron transport chain[10]. The counterintuitive corollary of this observation is that, in cells doomed to die, cytochrome *c* must leave the mitochondria for the cytosol (where the caspases reside). Counterintuitive this may be, but it appears to be true, at least in mammalian cells (reviewed in refs 5, 11).

In their paper, Zou *et al.*[2] present the sequence of Apaf-1, a protein of M_r 130,000. Remarkably, part of it shows a striking similarity to that of CED-4, with the two proteins aligning over most of the CED-4 sequence, including the nucleotide binding site. Flanking the CED-4 homology region are two other domains that provide additional hints as to how the Apafs might direct caspase activation (Fig. 1b). The amino terminus of Apaf-1 shows some sequence similarity to the prodomain of CED-3, and probably constitutes a caspase-recruitment (CARD) domain. CARD domains are found in a number of cell death proteins (including CED-3 and CED-4) and may bind directly to caspases[12]. This domain might thus be the link between the Apafs and caspase-3. At the carboxy terminus of the protein is a large domain containing 12 WD-40 repeats. Such repeats usually mediate protein–protein interactions, and could in this case be involved in the physical interaction that Zou *et al.*[2] observed between Apaf-1 and Apaf-2/cytochrome *c*.

So what does it all mean? That both CED-4 and Apaf-1 promote the activation of caspases suggests that their molecular mechanism of action is likely to be the same. However, as is the case with CED-9 versus Bcl-2, and CED-3 versus the caspases, regulation of Apaf-1 is likely to be more complex (or sophisticated, depending on your point of view), not least because mammalian cells must integrate many more signals before deciding whether to live or die.

Plenty of issues remain. Is Apaf-1, like CED-4, regulated by interaction with Bcl-2 family members? If so, does it show any preferences in its dealings with the various factions within the family? What are we to make of the WD-40 repeats in Apaf-1, and of their absence in CED-4? If they bind to cytochrome *c* (and possibly Apaf-3), does binding result in activation, or in relief of an intrinsic inhibitory activity? And are we to conclude that, in *C. elegans*, cytochrome *c* is not involved in cell death?

Finally, does Apaf-1 constitute the only mechanism that leads to caspase-3 activation? In *C. elegans*, this is clearly the case, because in the absence of CED-4 there is no cell death whatsoever. Such a dramatic effect seems unlikely in mammals, be it because of alternative activation pathways or redundancy with other caspases. Indeed, what about the other caspases? Do they each have their own Apaf-1 homologue, do they share a common activator, or are they simply activated by other caspases (through the fabled caspase cascade)? Questions, questions — much work remains to be done. □

Michael O. Hengartner is at the Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA.

e-mail: hengartn@cshl.org

1. Hengartner, M. O. & Horvitz, H. R. *Curr. Opin. Gen. Dev.* **4**, 581–586 (1994).
2. Zou, H., Henzel, W. J., Liu, X., Lutschg, A. & Wang, X. *Cell* **90**, 405–413 (1997).
3. Chinnaiyan, A. M., Chaudhary, D., O'Rourke, K., Koonin, E. V. & Dixit, V. M. *Nature* **388**, 728–729 (1997).
4. Seshagiri, S. & Miller, L. K. *Curr. Biol.* **7**, 455–460 (1997).
5. Shaham, S. & Horvitz, H. R. *Genes Dev.* **10**, 578–591 (1996).
6. Jacobson, M. D. *Curr. Biol.* **7**, R277–R281 (1997).
7. Reed, J. C. *Nature* **387**, 773–776 (1997).
8. James, C., Gschmeissner, S., Fraser, A. & Evan, G. I. *Curr. Biol.* **7**, 246–252 (1997).
9. Chinnaiyan, A. M., O'Rourke, K., Lane, B. R. & Dixit, V. M. *Science* **275**, 1122–1126 (1997).
10. Liu, X., Kim, C. N., Yang, J., Jemmerson, R. & Wang, X. *Cell* **86**, 147–157 (1996).
11. Golstein, P. *Science* **275**, 1081–1082 (1997).
12. Hofmann, K. *et al. Trends Biochem. Sci.* **22**, 155–156 (1997).

Microporous solids

Cobalt caged for catalysis

Robert L. Bedard

On page 735 of this issue[1], Stucky *et al.* introduce a new method of making microporous materials that contain transition metals. They demonstrate it by synthesizing cobalt phosphates and cobalt metal phosphates, and the family of materials that can be made in his way should include a large variety of compositions and crystalline structures, with the potential for new catalytic reactions

In most cases, the new cobalt phosphate structures have been observed before in the aluminosilicate zeolites, a class of materials that are not only synthesized industrially as catalysts and adsorbents, but also formed in nature by the weathering of volcanic rock and marine sediments. Similar structural mimicry of these naturally occurring minerals has been observed before in beryllium[2] and zinc[3] phosphate frameworks, but its extension into the transition elements is of interest because of their greater variety of useful chemical properties. With many oxidation states so easily accessible, transition metals will allow a wider range of reactions than the solid-acid chemistry of traditional zeolites—they should be able to catalyse oxidation and reduction reactions for example. The magnetic behaviour of the transition metals might also be exploited to create new types of molecule-selective devices.

The commercial applications of microporous materials are continually expanding. Materials of this type are often referred to as frameworks, to describe their scaffold-like, rigid crystalline structures; or as molecular sieves, because their pore cross-sections are of molecular dimensions. As well as the aluminosilicate zeolites, there are titanium silicates, aluminium phosphates, metal sulphides and selenides, transition-metal oxides, and many others.

These materials are used as catalysts in refining gasoline and diesel fuel; they aid powdered laundry detergents by softening water; and they can efficiently separate air into its valuable pure components, N_2 and O_2. Newer applications include the use of the molecule-sized cavities within these materials to carry out the selective oxidation of simple organic compounds into useful chemicals[4]. Microporous oxides, used as ion exchangers to selectively trap the highly radioactive isotopes ^{137}Cs and ^{90}Sr, are likely to play a leading role in cleaning up the nuclear waste generated by weapons programmes and nuclear power generation[5,6].

The challenge in synthesizing new frame-

Apoptosis

Death cycle and Swiss army knives

Michael O. Hengartner

Cytochrome *c* leads a double life. When a cell is called on to commit apoptotic suicide, cytochrome *c* relocalizes from the mitochondria to the cytosol. There, it helps to activate the foot-soldiers of apoptosis — the death proteases known as caspases[1]. How cytochrome *c* escapes from the mitochondria is still a matter of debate[1], but it is clear that certain elements within the apoptotic regulatory hierarchy do not condone such behaviour. In particular, overexpression of the cell-death suppressors Bcl-2 and Bcl-xL prevents the release of cytochrome *c*, suggesting that these proteins act upstream of cytochrome *c* in the pathway to cell death[2,3]. However, on pages 449 and 496 of this issue, Zhivotovsky *et al.*[4] and Rossé *et al.*[5] show that Bcl-2 can also protect cells downstream of cytochrome *c* release, forcing a re-evaluation of this newly acquired dogma.

Caspases do the brunt of the work in apoptosis. Activated through proteolytic processing, they cleave a limited number of apoptotic substrates and cause, directly or indirectly, most of the changes that are characteristic of apoptosis (for example, see ref. 6). So how are caspases activated? In the nematode worm *Caenorhabditis elegans*, the caspase homologue CED-3 is activated through a physical interaction between proCED-3 and CED-4. Interaction with CED-4 seems to be the only mechanism available for CED-3 activation, because there is no programmed cell death in *ced-4* mutant animals. In cells that should survive, CED-9 (which belongs to the Bcl-2 family) binds to CED-4 and holds it in an inactive conformation, thereby preventing CED-4-mediated activation of proCED-3. The ability of CED-9, CED-4 and CED-3 to exist in a multiprotein complex has led to the 'apoptosome' model of cell-death regulation (reviewed in ref. 7).

A series of elegant papers by Wang and colleagues indicated that at least some caspases are activated through a similar mechanism in mammals. This group purified, from human cell extracts, three proteins that can activate procaspase-3 in the presence of dATP. The molecular nature of two of these apoptotic protease activating factors — or Apafs — made perfect sense: Apaf-3 turned out to be caspase-9, which has a similar structure to CED-3 (ref. 8); and Apaf-1 was the long-sought mammalian homologue of CED-4 (ref. 9). As expected from sequence similarities with the worm proteins, Apaf-1 binds to Apaf-3/caspase-9 and promotes its proteolytic activation.

But there are two interesting twists to this story. First, unlike CED-4, Apaf-1 needs a cofactor in order to bind to and activate caspase-9. To general stupefaction, this cofactor — Apaf-2 — turned out to be the humble cytochrome *c*, which is normally present on the outer surface of the inner mitochondrial membrane. Here, it usually shuttles electrons between systems III and IV of the mitochondrial electron-transport chain.

The second twist concerns the mechanism of cell-death suppression by the Bcl-2 oncoprotein (and its homologues). Bcl-2 interacts with at least half a dozen proteins (not counting other Bcl-2-family members[10]), but it is still not clear how it prevents death. Although it would be attractive to use the *C. elegans* data to postulate that Bcl-2 will bind to Apaf-1 and/or other adaptors of its ilk, evidence for such a model is woefully lacking. Rather, last year two groups[11,12] showed that high levels of Bcl-2 can prevent the release of cytochrome *c* by mitochondria, providing an attractive biological — if not molecular — function for the protein (Fig. 1a). Could Bcl-2, prodded perhaps by the recruitment in mammals of a new apoptotic cofactor, have adopted a new function? Has the *C. elegans* model reached the limits of its usefulness?

Maybe not. The studies of Rossé *et al.*[5] and Zhivotovsky *et al.*[4] now suggest a more complicated picture. Using two different approaches, these groups find that high levels of Bcl-2 can delay death, even when cytochrome *c* is already in the cytosol. Rossé *et al.* used transient transfection of Bax, a pro-apoptotic member of the Bcl-2 family, to bring about cytochrome-*c* release and apoptosis. Co-transfection of Bcl-2 prevented Bax-induced apoptosis, yet cytochrome *c* nonetheless transferred to the cytosol. So, at least in this case, Bax seems to act upstream of cytochrome *c*, which in turn acts upstream of Bcl-2. Zhivotovsky *et al.* used a more direct, but also more invasive, approach — they microinjected high concentrations of cytochrome *c* into the cytosol. They found that high levels of Bcl-2 block most of the deaths, indicating that Bcl-2 can act downstream of cytochrome *c*.

According to the *C. elegans* model of CED-9/CED-4/CED-3, this is exactly what we might have expected. But how do we explain the finding that the same protein can block death at two distinct steps in the apoptotic pathway? One possibility is that Bcl-2 is just very resourceful, and simultaneously performs many functions (the Swiss army knife model[10]; Fig. 1a). Or maybe the two activities are linked. For example, Bcl-2 has been reported to undergo a large-scale conformational change that can lead to its insertion into membranes and, possibly, the formation of Bcl-2 channels[10]. Such an insertion could allow the release of cytochrome *c* (by hampering the formation of Bcl-2/Bax heterodimers?), and prevent Bcl-2 from binding to Apaf-1. If not all of the Bcl-2 molecules insert, then the remaining ones could still bind Apaf-1 and prevent cell death. This might be particularly apparent if the apoptotic pathway is stimulated downstream of the events that usually lead Bcl-2 to insert into the membrane.

Alternatively, Bcl-2 might seem to act both up- and downstream of cytochrome-*c*

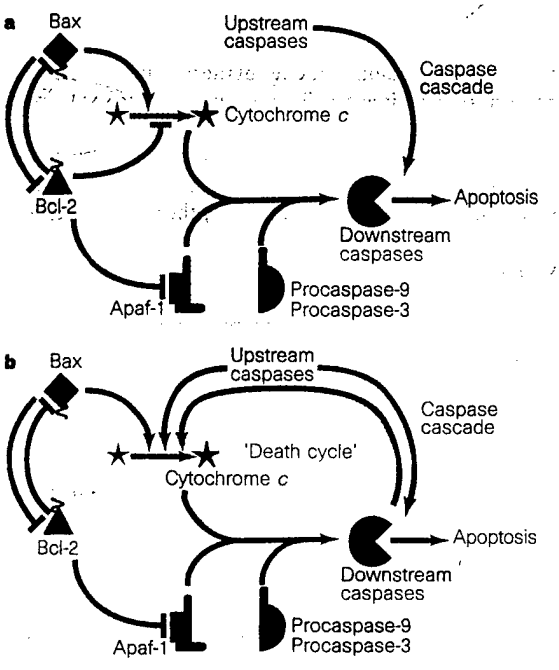

Figure 1 **Models for the function of Bcl-2 in apoptosis. Based on the genetic similarity with CED-9 from *C. elegans*, both models incorporate the idea that Bcl-2 can prevent apoptosis by interacting with caspase activators. a, The 'Swiss army knife' model[10]. Bcl-2 prevents the release of cytochrome *c* (perhaps by antagonizing Bax), as well as caspase activation in the presence of cytosolic cytochrome *c*. b, The 'death cycle' model. Bcl-2 blocks caspase activation, preventing amplification of pro-apoptotic signals. Signals that generate high levels of active caspases might be able to bypass the amplification step and activate the downstream caspases through a direct caspase cascade.**

release because it is involved in a circular pathway. Indeed, not only does cytochrome *c* stimulate caspase activation, but active caspases promote the release of cytochrome *c* from intact mitochondria. This suggests that mitochondria carry a caspase substrate that, when cleaved, promotes cytochrome-*c* release. Thus, mitochondria might act as apoptotic amplifiers, fostering a positive-feedback loop between cytochrome-*c* release and caspase activation (Fig. 1b). Any event that primes the loop — cytochrome-*c* release, caspase activation or otherwise — will initiate the vicious 'circle of death', eventually leading to large-scale caspase activation and apoptotic death.

For the death-cycle model to be viable, we need to add dampeners to the system. Otherwise, the slightest perturbation could be amplified, leading to unwarranted death. Good candidates are the inhibitors of apoptosis (IAP) family of proteins, which specifically inhibit particular caspases[13]. Bcl-2 could also be considered as a dampener, its role being to break the cycle — if the *C. elegans* model is right — by preventing caspase activation. If the cycle is broken early on, as might be the case with weak signals, there will be little release of cytochrome *c*, and Bcl-2 will seem to have acted upstream of cytochrome *c*.

In theory, Bcl-2 should also prevent strong pro-apoptotic signals, as long as they enter the cycle at the level of cytochrome-*c* release. But it might be a pyrrhic victory — such a cell might be technically alive, but if its mitochondria are mangled and its electron-transport chain disrupted, it is unlikely to thrive or divide. Neither Rossé *et al.*[5] nor Zhivotovsky *et al.*[4] report on the long-term prospects for the cells with cytosolic cytochrome *c*, leaving this question open.

At this point, both of the models can account for most of the published observations. Unfortunately, the proliferation of arrows (Fig. 1) makes falsification difficult, thereby limiting the predictive value of the models. In fact, probably the only safe prediction is that apoptosis will be a very complex process or, worse, a nonlinear one. Or both. □

Michael O. Hengartner is at Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA.
e-mail: hengartn@cshl.org

1. Reed, J. C. *Cell* **91**, 559–562 (1997).
2. Golstein, P. *Science* **275**, 1081–1082 (1997).
3. Duckett, C. S. *et al. Mol. Cell. Biol.* **18**, 608–615 (1998).
4. Zhivotovsky, B., Orrenius, S., Brustugun, O. T. & Døskeland, S. O. *Nature* **391**, 449–450 (1998).
5. Rossé, T. *et al. Nature* **391**, 496–499 (1998).
6. Enari, M. *et al. Nature* **391**, 43–50 (1998).
7. Hengartner, M. O. *Nature* **388**, 714–715 (1997).
8. Li, P. *et al. Cell* **91**, 479–489 (1997).
9. Zou, H., Henzel, W. J., Liu, X., Lutschg, A. & Wang, X. *Cell* **90**, 405–413 (1997).
10. Reed, J. C. *Nature* **387**, 773–776 (1997).
11. Yang, J. *et al. Science* **275**, 1129–1132 (1997).
12. Kluck, R. M., Bossy-Wetzel, E., Green, D. R. & Newmeyer, D. D. *Science* **275**, 1132–1136 (1997).
13. Deveraux, Q. L., Takahashi, R., Salvesen, G. S. & Reed, J. C. *Nature* **388**, 300–304 (1997).

Axon guidance

A Roundabout way of avoiding the midline

Barry Dickson

Bilaterally symmetrical animals must be able to integrate sensory inputs and coordinate motor control on both sides of the body. Thus, many neurons in the central nervous system (CNS) project their axons to the opposite side of the body, whereas others project axons that remain on the same side. In the latest issues of *Cell*[1,2] and *Neuron*[3], the groups of Corey Goodman, Guy Tear, Marc Tessier-Lavigne and Cori Bargmann report that, from worms and flies to rats and humans, a common mechanism determines which axons cross the midline and which do not.

In insects and vertebrates, the two symmetrical halves of the CNS are separated by a specialized group of cells. Located at the ventral midline of the CNS, these are called midline cells in insects, and they form the floor plate in vertebrates. Most CNS axons initially grow towards these midline cells and then turn longitudinally — either on their own side (ipsilateral), or by first crossing the midline and then turning (contralateral). As they project longitudinally, these axons skirt along the edge of the midline, but never cross it again. Those axons that cross the midline form the commissures that allow sensory information and motor instructions to pass from one side of the animal to the other.

A few years ago, there was much excitement when midline cells in worms, flies and vertebrates were all found to secrete the same type of chemoattractants, netrins, to guide axons along the initial part of their trajectories towards the midline[4–8]. But evidence has been accumulating from studies in the fly[9], grasshopper[10], fish[11] and chick[12] suggesting that the midline is not only the source of a long-range attractive signal, but also of a short-range repulsive signal. Kidd *et al.*[1] and Zallen *et al.*[2] now identify a family of putative receptors for such a repulsive signal — the Roundabout (Robo) family. So far, one *robo* gene has been identified in worms, two in flies, two in the rat and two in humans, suggesting that the short-range repulsive signal has been just as highly conserved during the course of evolution as the long-range attractive signal.

Mutations in the *robo* gene were originally recovered in a screen for abnormal axon-projection patterns in the *Drosophila* CNS[9]. In *robo* mutant embryos, axons meander back and forth across the midline. Mutations in *sax3* — a closely related *Caenorhabditis elegans* gene — result in a similar phenotype[2]. The worm ventral nerve cord is asymmetrical but, nevertheless, it is divided into discrete left and right axon bundles. Axons mainly cross from left to right only at the anterior and posterior ends of the nerve cord. In *sax3* mutants, however, axons cross the midline in either direction along the length of the nerve cord. Kidd *et al.* and Zallen *et al.* have also found that the *robo* and *sax3* genes encode immunoglobulin-type transmembrane receptors, which are expressed on neuronal growth cones as they encounter the midline[1,2]. One of the rat *robo* genes is also expressed by spinal-cord neurons when their axons are responding to guidance cues from the floor plate. So rat *robo*, like the invertebrate *robo* genes, may be reading a signal that prevents these axons from repeatedly crossing the midline[1].

A combination of a long-range attractant and short-range repellent expressed by the same midline cells would elegantly explain why most axons first project towards the midline, and, on reaching it, turn to follow a parallel pathway, never straying across the midline or back towards the periphery. But why do most axons cross the midline before making this turn? And why do they cross only once? Conceivably, crossing and non-crossing axons might differ in their responses to the attractive or the repulsive cue.

It is not yet clear whether netrins are also involved in the crossing decision, because it is experimentally difficult to separate guidance towards and across the midline. For example, the partial loss of commissures in *netrin* mutant fly embryos[7,8] may occur because many commissural axons fail even to make contact with midline cells. Still, netrins are not the only attractive signals provided by the midline and floor plate, and commissural and longitudinal axons may differ in their sensitivity to some of these other cues.

Differential sensitivity to the midline repellent, at least in flies, is dramatically shown by Kidd *et al.*[1]. The Robo protein is expressed on the growth cones of the longitudinal axons, yet it is almost completely absent from commissural axons — until they have crossed the midline. This explains why commissural (but not longitudinal) axons can cross the midline, and also why, having crossed once, commissural axons never turn back and cross again.

In a companion paper, Kidd *et al.*[3] show that Robo is downregulated by a transmembrane protein encoded by the *commissureless* (*comm*) gene. As the name implies, axons